CHAPTER

4

Shaw-*Related* K^+ *Channels in Mammals*

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I. Introduction

Twenty-five transcripts from genes related to the *Drosophila Shaker* gene (the *Sh* gene family) have been identified in mammals by molecular methods. *Sh* transcripts are thought to encode subunits of tetrameric voltage-gated K^+ channels. However, the relationship between native K^+ channels and the molecular components identified by cloning, and thereby the physiological significance of this molecular diversity, remain to be elucidated.

Sh genes and their products can be divided into four subfamilies. Compelling evidence suggests that subunits of the same subfamily, but not of different subfamilies, form heteromultimeric channels *in vitro;* thus, each gene subfamily is postulated to encode components of an independent channel system. The potential significance of heteromultimer formation demonstrates the importance of studying the genes of one subfamily as a group when the physiological role of any of these genes is being pursued. This chapter focuses on the characterization of the *Shaw*-related or *Sh*III subfamily in mammals, emphasizing the work done to understand the relationship between cloned components and native K^+ channels. This work illustrates how molecular biology may contribute to the discovery of new, previously unidentified K^+ channels.

II. Molecular Diversity and Neuronal Function

Diversity is an old and recurring theme in the organization and function of the nervous system. The early neuroanatomists recognized that the large variety of neuronal forms must be associated with functional specialization. The application of intracellular recording techniques to the nervous system several decades later revealed a similar diversity of intrinsic electrophysiological properties of neurons. This theme is re-emerging in the study of the molecules responsible for the generation and transmission of signals in neurons.

Large numbers of protein components of ligand- and voltage-gated ion channels as well as neurotransmitters and neuropeptide receptors (which modulate the activity of ion channels through intermediary second messenger cascades) are being discovered by molecular cloning (Betz, 1990,1992; Cockroft *et al.*, 1990; Buck and Axel, 1991; Catterall, 1992; Gasic and Hollmann, 1992; Hille, 1992; Nakanishi, 1992; Neher, 1992; Role, 1992; Sommer and Seeburg, 1992; Wisden and Seeburg, 1992; Jessell and Kandel, 1993; Sargent, 1993). This diversity suggests a degree of functional specificity well beyond that previously considered and that the power of generating variation by gene duplication and divergence played a role in the gener-

ation of complexity in the nervous system of animals with elaborate nervous systems. These studies will influence our views on the organization and function of the nervous system.

Much of the cloning work has been done in the absence of prior isolation of native proteins. Therefore, the exact relationship between the molecular components identified by cloning and the native channels or receptors is, in most cases, not known. Therefore, the physiological significance of the molecular diversity that is being discovered remains to be elucidated.

One notable example is the large number of subunits of γ-aminobutyric acid ($GABA_A$) receptors that has been cloned (reviewed by Seeburg *et al.*, 1990; Wisden and Seeburg, 1992; see also Chapter 21). Since $GABA_A$ receptors are thought to be pentameric, the variety of receptors that could be generated from different combinations of these subunits is truly staggering, suggesting variations in synaptic inhibition that were not previously expected. New views on neuronal integration are likely to emerge from understanding the physiological significance of this molecular diversity. Although the problem is complex, progress is being made in determining which combinations of subunits may exist *in vivo* and where they might be present (Wisden and Seeburg, 1992), in establishing functional differences between receptors with different subunit combinations reconstituted in model expression systems (Seeburg *et al.*, 1990; Sigel *et al.*, 1990; Verdoorn *et al.*, 1990), and in determining the possible roles of specific isoforms in brain function (Korpi *et al.*, 1993). A similar problem exists for the components of K^+ channels that have been identified by cloning methods.

III. Families of K^+ Channel Genes

K^+ channels, a group of ligand- and voltage-gated channels that conduct K^+ ions preferentially, are present in most, if not all, eukaryotic cells. These channels play key roles in a wide range of cellular functions including excitability, secretion, differentiation, mitogenesis, and osmotic regulation (e.g., Thompson and Aldrich, 1980; Klein *et al.*, 1982; Llinas, 1984,1988; Peterson and Maruyama, 1984; Adams and Galvan, 1986; Kaczmareck and Levitan, 1987; Levitan, 1988; Lewis and Cahalan, 1988; Rudy, 1988; Latorre *et al.*, 1989; Grinstein and Smith, 1990; Hille, 1992; Dubois and Rouzaire-Dubois, 1993). Among the ion channels, K^+ channels constitute a group exhibiting particularly large functional diversity (Rudy, 1988; Latorre *et al.*, 1989; Hille, 1992); K^+ channel types vary in their response to factors, such as intracellular Na^+ and Ca^{2+} concentrations and the membrane potential, that determine the opening and closing of the channel. K^+ channels also vary in kinetics, permeability properties, and sensitivity and type of response to toxins, drugs, and modulating factors such as second messenger cascades. This diversity allows a fine tuning of resting membrane potentials and action potential waveforms, and a modulation of firing and neurotransmitter-secretion patterns (Klein *et al.*, 1982; Kaczmareck and Levitan, 1987; Rudy, 1988; Levitan, 1988; Llinas, 1988; McCormick, 1990; Baxter and Byrne, 1991; Hille, 1992). Since K^+ channels are frequently targets of second messenger cascades, their diversity influences the type of cellular response to different types of stimuli (Kaczmareck and Levitan, 1987; Levitan, 1988; Lopez-Barneo *et al.*, 1988; Rudy, 1988; Hille, 1992). Alterations in excitability may arise by differential spatiotemporal expression of K^+ channels.

Molecular cloning of K^+ channel components is an enterprise that began with the cloning of the *Shaker* locus in *Drosophila* (Baumann *et al.*, 1987; Kamb *et al.*, 1987; Papazian *et al.*, 1987; Tempel *et al.*, 1987). Subsequent work has revealed a molecular diversity of K^+ channel components that parallels the functional diversity of K^+ channels *in vivo*. Over 30 different cDNAs corresponding to transcripts from several gene families have already been discovered (Fig. 1). Of these families, the one consisting of homologs of the *Drosophila Shaker* gene (called here the *Shaker* or *Sh* gene family) encoding putative subunits of voltage-gated K^+ channels (reviewed in Jan and Jan, 1990a; Perney and Kaczmareck, 1991; Rehm and Tempel, 1991; Rudy *et al.*, 1991a; Pongs, 1992) has been studied most extensively and is the subject of this review.

A. Sh *Proteins: Putative Subunits of Voltage-Gated* K^+ *Channels*

Nearly 20 *Sh* genes have been identified in mammals. Based on sequence similarities and, hence, probable evolutionary relationships, the family is divided into four groups or subfamilies (reviewed by Jan and Jan, 1990a; Perney and Kaczmareck, 1991; Rudy, *et al.*, 1991a; Pongs, 1992; Salkoff *et al.*, 1992; see also Chapter 1) designated here as *Sh*I, *Sh*II, *Sh*III and *Sh*IV. A member of a subfamily in mammals is also more similar to one of four related *Drosophila* genes (*Shaker, Shab, Shaw,* and *Shal;* Butler *et al.*, 1989; Wei *et al.*, 1990) than to a mammalian member of a different subfamily, suggesting that these subfamilies are derived from precursors that existed prior to the divergence of mammals and insects. *Sh*I mammalian genes are thought to be homologs of *Shaker, Sh*II of

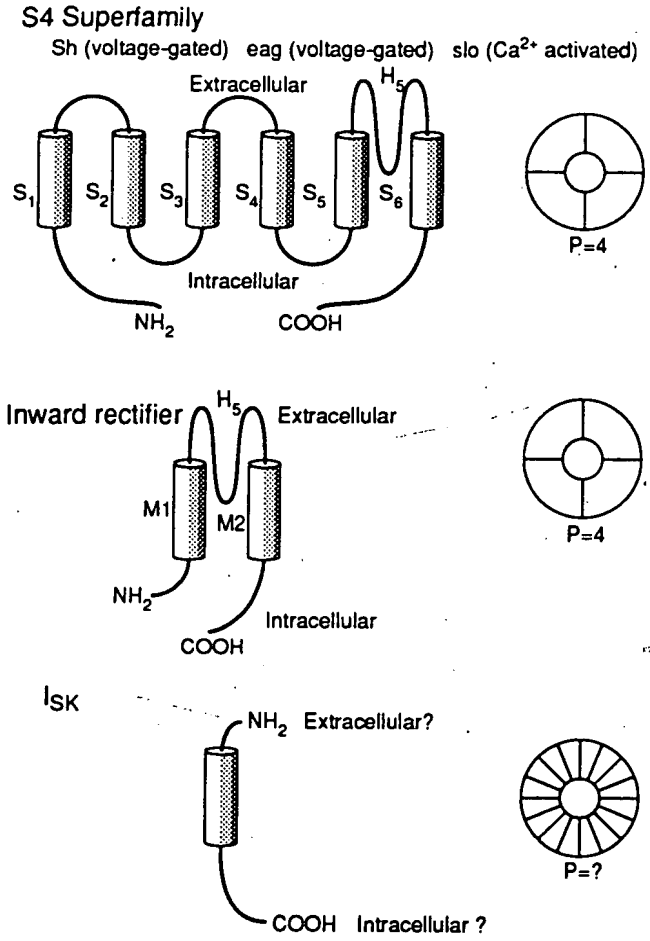

FIGURE 1 Families of K^+ channel subunits. Three different types of protein subunits of K^+ channels are shown. (*Top*) K^+ channel proteins of the S4 superfamily include those of the *Sh* and *eag* families, which are components of voltage-gated K^+ channels, as well as those of the *slo* family, which are components of calcium-activated K^+ channels. This superfamily also includes cyclic nucleotide-gated channels and the α subunits of voltage-gated Na^+ and Ca^{2+} channels (Jan and Jan, 1990b). K^+ channel proteins of the S4 superfamily and cyclic nucleotide-gated channel proteins contain six membrane spanning domains (including an S4 domain) and a pore domain (with an H5-like sequence on K^+ channels); four such polypeptides form one functional channel. α subunits of voltage-gated Na^+ and Ca^{2+} channels consist of four homologous domains, each resembling a K^+ channel or cyclic nucleotide-gated channel protein (Catterall, 1988). (*Middle*) Inward rectifier K^+ channel proteins, also proposed to form tetrameric channels (Kubo *et al.*, 1993), contain two membrane spanning domains and one H5-like pore domain. (*Bottom*) The I_{SK} protein expresses slowly activating voltage-dependent K^+ currents and contains a single membrane spanning domain and no H5 domain. How many such subunits form a functional channel is not known (Takumi *et al.*, 1988).

Shab, *Sh*III of *Shaw*, and *Sh*IV of *Shal*. Multiples genes of each subfamily have been described in mammals (*Sh*I, 7; *Sh*II, 2; *Sh*III, 4; and *Sh*IV, 3), indicating that, in the chordate line (Deuterostomia), the precursors underwent extensive duplication and subsequent variation, leading to a large number of closely related genes. In addition, two *Sh* cDNAs isolated from rat libraries may define two additional subfamilies (Drewe *et al.*, 1992). Moreover, mammalian *Sh*III genes encode more than one product by the process of alternative splicing (Luneau *et al.*, 1991a,b; Rudy *et al.*, 1992; Vega-Saenz, de Miera *et al.*, 1992), leading to the existence of 25 known *Sh* transcripts. All these transcripts are expressed in brain, and some are expressed in other tissues as well.

Each *Sh* transcript induces the expression of voltage-dependent K^+ currents with characteristic voltage dependence and kinetic and pharmacological properties when introduced in heterologous expression systems such as *Xenopus* oocytes and cells in culture (Jan and Jan, 1990a; Perney and Kaczmareck, 1991; Rudy *et al.*, 1991a; Pongs, 1992; Salkoff *et al.*, 1992). Evidence strongly suggests that the channels underlying these currents are homomultimers (Jan and Jan, 1990a; Perney and Kaczmarek, 1991; Rudy *et al.*, 1991a; Pongs, 1992, Salkoff *et al.*, 1992), probably tetramers of *Sh* proteins (MacKinnon, 1991a; Liman *et al.*, 1992). Therefore, the proteins encoded by *Sh* transcripts are likely to be subunits of voltage-gated K^+ channels found in neurons and other cells. One of the current challenges is understanding the functional roles of the cloned products, beginning with the identification of native voltage-gated K^+ channels that contain these components.

B. Heteromultimer Formation and Channel Subunit Composition

Compelling evidence exists that *in vitro* coexpression of different subunits from the same subfamily, *but not from different subfamilies*, results in the formation of electrophysiologically distinct heteromultimeric channels (Christie *et al.*, 1990; Isacoff *et al.*, 1990; K. McCormack *et al.*, 1990; Ruppersberg *et al.*, 1990; Covarrubias *et al.*, 1991; Weiser *et al.*, 1993). The significance of heteromultimer formation in determining channel composition *in vivo* is still not fully appreciated. Other factors, such as interaction with non-*Sh* subunits and posttranslational modifications, may also contribute to the functional properties of native channels containing *Sh* proteins (Rehm and Lasdunzky, 1988; Rehm *et al.*, 1989a,b; Rudy *et al.*, 1988,1991a; Warmke *et al.*, 1991; Zhong and Wu, 1991). Further, the influence of heteromultimer formation, interaction with non-*Sh* subunits, and posttranslational modifications could vary from cell to cell. K^+ channel diversity arising from these factors may contribute to the individualities in electrophysiological properties of single neurons in the nervous system.

Given the number of subunits available and of factors that could determine channel composition *in vivo*, the task of understanding the relationship between the diversity of molecular components and the functional diversity of K^+ channels is far from simple. These problems are symptomatic of the challenge that follows the isolation of cloned gene products: the right pieces are available but we must discover what they do. This chapter focuses on work that addresses this problem for the products of the *Sh*III or *Shaw*-related subfamily of K^+ channel genes.

IV. Toward an Understanding of the Functional Role of Mammalian *Shaw*-Related Proteins

A. *Four* Shaw-*Related Genes Encode at Least 11 Transcripts*

As in the other mammalian *Sh* subfamilies the *Shaw*-related or *Sh*III subfamily consists of several genes formed by gene duplication throughout animal evolution. However, this group is the only class of *Sh* genes in mammals for which evidence exists of alternative splicing also contributing to the generation of diversity. Researchers have isolated 11 different *Sh*III cDNAs from libraries derived from rodent and human mRNA (Yokoyama *et al.*, 1989; T. McCormack *et al.*, 1990,1991; Luneau *et al.*, 1991a,b; Rudy *et al.*, 1991b,1992; Sen *et al.*, 1991; Schroter *et al.*, 1991; Ghanshani *et al.*, 1992; Vega-Saenz de Miera *et al.*, 1992). Based on sequence analysis, these cDNAs are thought to represent transcripts from four different genes that generate more than one product by alternative splicing (Fig. 2).

Various names for the four *Sh*III genes and their products are shown in Table I. This table also includes the corresponding names in a standardized nomenclature proposed by several investigators in the field (Chandy *et al.*, 1991) and the name assigned to each gene by the Human Genome Nomenclature Committee. The nomenclature of Chandy *et al.* will be implemented throughout this chapter.

1. Evidence for the Existence of Four *Sh*III Genes

The nucleotide sequences of the cDNAs thought to be products of independent genes differ throughout the length of the cDNAs, suggesting that they are derived from transcripts of distinct genes rather than by alternative splicing. The analysis of genomic clones (see subsequent discussion) and the locations of *Sh*III genes in human and mouse chromosomes (Table II) are further evidence that the eleven transcripts are derived from four independent genes.

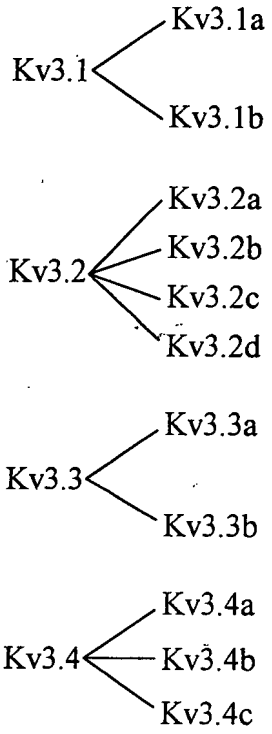

FIGURE 2 *Sh*III K^+ channel subunits. Four mammalian *Shaw*-related genes KV3.1, KV3.2, KV3.3, and KV3.4 encode 11 different K^+ channel subunit isoforms by alternative splicing.

TABLE I **Nomenclature of *Shaw*-Related K^+ Channel Transcripts in Mammals**

Name used in this chapter[a]	Other names	Gene symbol[b]
KV3.1a	NGK2, RKShIIIB, KShIIIB, KV3.1β	KCNC1
KV3.1b	KV4, KV3.1α, Raw2	
KV3.2a	RKShIIIA, (R)KShIIIA.1	KCNC2
KV3.2b	KShIIIA.3	
KV3.2c	—	
KV3.2d	KShIIIA.2, Raw1	
KV3.3a	KShIIID.1	KCNC3
KV3.3b	KShIIID.2	
KV3.4a	Raw3	KCNC4
KV3.4b	(H)(R)KShIIIC	
KV3.4c	—	

[a] Nomenclature based on Chandy *et al.* (1991).
[b] Symbol assigned to the gene by the human genome nomenclature committee.

TABLE II **Chromosomal Localization of *Sh*III Genes in Mice and Humans**

Gene	Chromosomal location (mice)	Chromosomal location (human)
KCNC1	7 (a)	11p15 (b,c)
KCNC2	10 (a)	12 (c); 19 (a)
KCNC3	10 (a); 7 (d)	19q13.3-4 (a,e)
KCNC4	NA	1p21 (e,f)

Mapping references are (a) Haas *et al.*, 1993; (b) Ried *et al.*, 1993; (c) Grissmer *et al.*, 1992; (d) D. Lau, V. Clarke, P. D'Eustachio, and B. Rudy, unpublished observations; (e) Ghanshani *et al.*, 1992; (f) Rudy *et al.*, 1991b.

NA, not available. The discrepancies in the locations of KCNC3 in mouse and KCNC2 in human remain to be clarified.

2. Alternative Splicing Generates Carboxyl-End *Sh*III Variants

Putative alternatively spliced transcripts of each gene have identical nucleotide sequences from the assumed starting ATG up to a point of divergence near, but prior to, the in-frame stop codon, predicting protein products with different carboxyl ends (Fig. 3). Mature transcripts from each gene diverge at the same point in the sequence, following an AG, the dinucleotide characteristic of the exonic portion of donor or 5′ splice junctions (Mount, 1982). These sequence relationships suggest that the different transcripts arise by alternative splicing of a primary transcript rather than by transcription from separate but highly homologous genes. In two cases, KV3.1 (Luneau *et al.*, 1991a) and KV3.2 (Luneau *et al.*, 1991b), support for the alternative splicing mechanism comes from analysis of rat genomic DNA in Southern blots. Probes derived from the sequence preceding the point of divergence detected a single restriction fragment for each gene in Southern blots of rat genomic DNA hybridized under high stringency conditions. This result indicates that the genes identified by those probes are represented only once in the rat genome.

B. Patterns of Alternative Splicing and Genomic Structure

Given the results of partial genomic analysis of the KV3.1 (Luneau *et al.*, 1991a), KV3.3 (Ghanshani *et al.*, 1992), and KV3.2 (D. Lau and B. Rudy, unpublished observations) genes, as well as analysis of cDNAs thought to be derived from incompletely processed RNAs (E. Vega-Saenz de Miera and B. Rudy, unpublished observations), all *Sh*III genes apparently have a similar structure. This hypothesis is consistent with the idea thal all four genes arose by duplication of a single ancestral gene. This possibility is further borne out by the patterns of alternative splicing of *Sh*III genes (Fig. 4).

As depicted in Fig. 4, the amino end of the proteins is encoded in a single exon (exon 1) that is constitutively spliced in the four genes to a downstream exon (exon 2) encoding most of the membrane core portion of the polypeptides,[1] from the beginning of the S1 domain to a point in the carboxyl end region following the S6 domain. Evidence that the amino end is encoded in a single exon comes from the analysis of rodent genomic clones of KV3.3 (Ghanshani *et al.*, 1992) and KV3.2 (D. Lau and B. Rudy, unpublished results). Exon 2 has been identified in genomic clones of KV3.1 (Luneau *et al.*, 1991a), KV3.2 (D. Lau and B. Rudy, unpublished results), and KV3.3 (Ghanshani *et al.*, 1992). The data are consistent with the presence of two sites of alternative splicing in *Sh*III genes: the first at the end of exon 2 and the second at the end of exon 3. In two of the four *Sh*III genes, KV3.1 (Luneau *et al.*, 1991a) and KV3.3 (Vega-Saenz de Miera *et al.*, 1992), the sequence immediately following exon 2 is either read through, generating KV3.1a and KV3.3b, or spliced out and replaced by exons present downstream to generate KV3.1b and KV3.3a. In KV3.1a and KV3.3b, the nucleotide sequence following the AG at the end of exon 2 starts with GT, the dinucleotide characteristic of the intronic portion of 5′ splice sites (Mount, 1982), supporting the idea that the divergent region of these transcripts is derived from the sequence following the splice site.

Genomic DNA analysis provides direct evidence for this "splice/don't splice" alternative splicing mechanism (McKeown, 1992). Utilizing polymerase chain reaction (PCR) of rat genomic DNA, Luneau *et al.* (1991a) demonstrated that the sequence encoding the divergent 3′ end of KV3.1a is contiguous with exon 2. These investigators also isolated a rat genomic clone that contains two exons encoding the divergent sequence of KV3.1b (exon 3 and exon 4). The 5′ end of exon 3 of the KV3.1 gene starts with the sequence of KV3.1b immediately following the point of divergence and extends 188 bp in the 3′ direction. Exon 4, separated from exon 3 by an intron, contains the remainder of the 3′ end of KV3.1b. In the case of the KV3.3 gene, Ghanshani *et al.* (1992) isolated a mouse genomic clone

[1] As described in Section IV,G, *Sh*III proteins (like other *Sh* proteins) consist of a core membrane region with six putative (S1, S2, S3, S4, S5, and S6) membrane-spanning domains flanked by putatively intracellular amino and carboxyl domains.

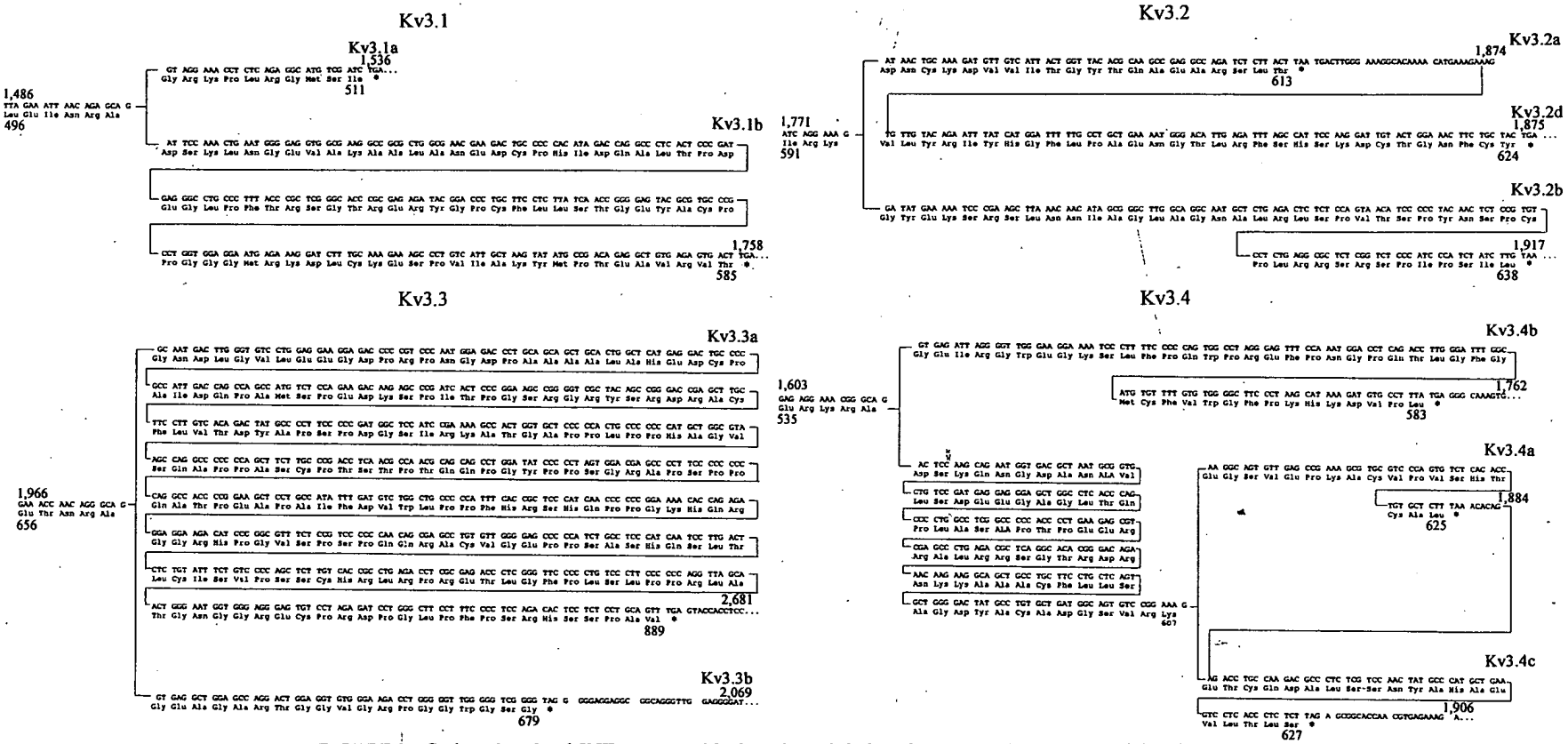

FIGURE 3 Carboxyl ends of *Sh*III proteins. Nucleotide and deduced amino acid sequences of the alternatively spliced 3′ ends of transcripts from four *Sh*III genes. In all cases, the nucleotide and predicted amino acid sequences diverge after an AG. The first in-frame stop condons are indicated with asterisks. The alternatively spliced carboxyl ends contain serines and threonines within consensus sequences for phosphorylation by various protein kinases (see Table IV).

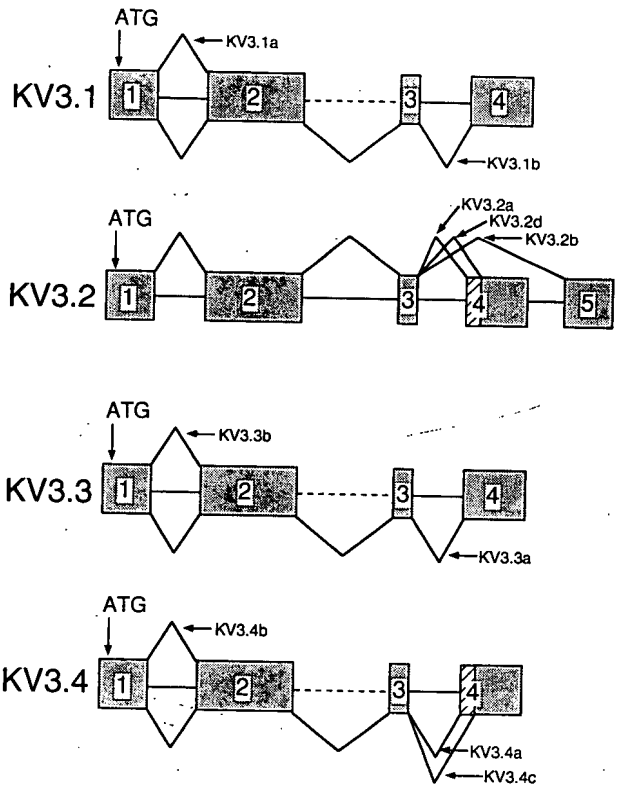

FIGURE 4 Genomic structure and patterns of alternative exon usage of four *Sh*III genes. In this schematic depiction of the structure of the coding regions of four *Sh*III genes, exons are indicated by numbered filled boxes, and introns are indicated by lines. Broken lines in KV3.1, KV3.3, and KV3.4 indicate sequences that are read through to generate one alternatively spliced transcript or spliced out and replaced by downstream exons to generate other mature transcripts. Exon 4 contains an internal acceptor site in KV3.2 and KV3.4. The region of the exon preceding this acceptor site is cross-hatched. Lengths of exons and introns are not necessarily to scale.

in which the divergent sequence of KV3.3b is contiguous with the sequence of exon 2. Although the exons encoding the carboxyl end of KV3.3a have not been identified, the structure of the KV3.3 gene in this region is suspected to be similar to that of the KV3.1 gene, based mainly on the sequence similarities between the divergent sequence of KV3.1b and KV3.3a transcripts (Vega-Saenz de Miera *et al.*, 1992), particularly in the region postulated to be encoded by exon 3 (Fig. 5).

In contrast to the KV3.1 and KV3.3 alternatively spliced variants, none of the divergent KV3.2 carboxyl ends identified to date begin with GT, suggesting that KV3.2 variants arise by a different splicing mechanism. Sequence comparisons also suggest that the site of alternative splicing in KV3.2 is different than that in KV3.1 and KV3.3. The region shared by all KV3.2 transcripts extends beyond a site equivalent in position to the end of exon 2 in KV3.1 and KV3.3 (indicated with an arrow in Fig. 11) by 165 bp. Moreover, this additional sequence shows significant similarity (Fig. 5) to exon 3 of the other genes. Therefore, alternative splicing of KV3.2 may take place at the end of exon 3 rather than at the end of exon 2.

No direct information is available on how the known KV3.2 variants are generated. However, sequence analysis suggests that the KV3.2a and KV3.2b variants arise from the use of alternative exons that follow exon 3 (Fig. 4). The number and position of these exons in genomic DNA might differ from that shown in Fig. 4. The sequence of KV3.2d, immediately following the splicing point, is found in KV3.2a 95 bp downstream (see Fig. 3). After this new overlap, the two sequences are identical. Thus, KV3.2d may arise by use of an internal acceptor site in the same exon used to generate KV3.2a, as suggested in Fig. 4. Luneau *et al.* (1991b) isolated a partial cDNA that may correspond to a fourth alternatively spliced KV3.2 transcript (KV3.2c). However, these investigators were unable to amplify the 5′ end of this cDNA with primers derived from the 5′ end of KV3.2a. Further characterization is required to understand whether this is a *bone fide* transcript of the KV3.2 gene and how it is generated.

Both alternative splice sites of *Sh*III genes (at the end of exon 2 and at the end of exon 3) described so far appear to be used to generate KV3.4 variants, although no genomic evidence exists in this case. KV3.4b, which has only been found in humans, diverges from KV3.4a and KV3.4c at the same point as KV3.1 and KV3.3 variants. Further, the nucleotide sequence of KV3.4b immediately after the divergence starts with GT, as in KV3.1a and KV3.3b. This observation is consistent with the hypothesis that KV3.4b arises by reading through the sequence immediately following exon 2 of the KV3.4 gene. KV3.4a and KV3.4c, which have only been identified in rat, have an identical 204-bp sequence following their divergence from KV3.4b. This sequence also shows significant sequence similarity with the sequence thought to derive from exon 3 in KV3.1b, KV3.2, and KV3.3a transcripts (Fig. 5). Thus, the alternative splicing in this case is likely to take place at the end of exon 3. The relationship between KV3.4a and KV3.4c is similar to the relationship between KV3.2a and KV3.2d. The sequence of KV3.4c immediately following the splice point is found 62 bp downstream in KV3.4a. After this new overlap, the two sequences are identical. These differences suggest an internal acceptor splicing mechanism, as in the case of KV3.2a and KV3.2d.

Except for KV3.3b and KV3.4c (which have not been tried) all alternatively spliced *Sh*III transcripts

A

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Kv3.1                                    ATTCCAAACTGAATGGGGAGGTGGCGAAGGCCGCGCTGGCGAACGAAGAC  1554
Kv3.2                      TGTTATCAGGTG-CGA--GTACAGGAA-T---                                  1647
Kv3.3  GCAATGACTTGGGTGTCCTGGAGGAAGGAG-CC--CGT-CC-----A--CCCT--AGCA--T--A-----TC-T--G---  2061
Kv3.4                                    -C-----G-A------T--C-   -T--T--G-T----T-CG-T--G--G  1665

Kv3.1  TGCCCCCACATAGACCAG                 GCCCTCACTCCCGATGAGGGCCTGCCCTTTACCCGCTCGGGCACCCGCGA  1622
Kv3.2                                 CCGC-AT-AT-A--TCCG--AA-G--C---A-C-GA-----TA-T---A-A--  1700
Kv3.3  ------GC---T------CCA             ---A-GT----A--A--CAAGAGC--GA-C--T   C-C--A-G---G-G  2129
Kv3.4  G-AG-TGG-C-CAC----CCCCTGGCCTCG----C---C--T--A---C-T-GAG--C-G-GA-----A-----A--G--  1745

Kv3.1  GAGATACGGACCC          TGCTTCCTCTTATCAACCGGGGAGTACGCGTGCCCGCCTGGTGGAGGAATGAGAAAG   1692
Kv3.2  C-A-A--A--AGAGGGGAAACA--T-----G--GA-G--A--T--T---A-----G-TT---A---------C--G--AG  1780
Kv3.3  TC-C---A-C-GGGACCGAGCT--------TG-CA--       --C--T--CCCTT-C--C-A---CTCC--CC----AG  2203
Kv3.4  C---A--AAGAAGGCAGCTGCC--------GC-CAGTG-T-----C--T--C--TG-TGA---CA-T-   -CCAG--AG  1822
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B

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Kv3.1          DSKLNGEVAKAALANEDCPHIDQ     ALTPDEGLPFTRSGTRERYGP   CFLLSTGEYACPPGGGMRK  564
Kv3.2      VLSGD--TGSEP                    P-S-P-R--IR--S--DKNRRGET----T--D-T-ASD-=I--  593
Kv3.3  GNDLGVLEEG-PRP--DP-A----H----A---P   -MS-EDKS-I-PGS -G--SRDRA---VT  D--PS-D-SI--  734
Kv3.4          ---Q--D -N-V-SD-EGAGLT-PLAS-P--E-RRALR-----D-NKKAAA-----A-D---A D-SV--  607
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FIGURE 5 Sequence of putative exon 3 of four *Sh*III genes. (A) The nucleotide sequence of exon 3 of the KV3.1 gene is compared with the last 165 bp of the constant region of KV3.2, the first 222 bp of the divergent end of KV3.3a, and the first 204 bases of the divergent ends of KV3.4a and KV3.4c. In all cases, the sequence shown is preceded by AG. The sequences are aligned to maximize long stretches of identity. Nucleotides identical to those in KV3.1 are shown with a dash. Gaps required for optimized alignment of the sequences are shown as blanks. (B) Alignment of the amino acid sequences encoded by the putative exon 3 of each gene. Amino acid residues identical to those in KV3.1 are shown with a dash. Gaps required for optimized alignment of the sequences are shown as blanks.

identified here are found in brain poly(A)-selected mRNA and express currents in *Xenopus* oocytes (Yokoyama *et al.*, 1989; T. McCormack *et al.*, 1990; Luneau *et al.*, 1991a,b; Rudy *et al.*, 1991b,1992; Schröter *et al.*, 1991; Vega-Saenz de Miera *et al.*, 1992; Weiser *et al.*, 1994). These results support the notion that these cDNAs represent real transcripts rather than the result of priming of unprocessed pre-mRNA.

1. Alternative Splicing of 5′ UTR

The previous section discussed alternative splicing of *Sh*III genes 3′ to the putative initiation codon. However, Kentros *et al.* (1992) have isolated cDNA clones of KV3.2 containing two different 5′ untranslated regions (5′UTRs) as well, which they have designated α and β. Northern blot analysis of rat brain poly(A) RNA with probes specific to these two 5′ UTRs shows hybridization to the major bands seen with coding region probes, whereas PCR experiments with primers specific to both α and β show that both 5′ UTRs can be associated with more than one of the various 3′ coding region, alternatively spliced variants of KV3.2. These experiments suggest that both 5′ UTRs are not simply the result of unspliced RNA. Alternative splicing of the 5′ UTR was also reported for KV1.1 a Shaker-related gene (Tempel *et al.*, 1988).

C. *Multiple Transcripts of Each* Sh*III Gene Are Found in Brain mRNA*

Northern blot analysis of rat brain poly(A) RNA shows that all members of the *Sh*III subfamily have multiple transcripts (Fig. 6). Specifically, KV3.1 probes hybridize to bands of approximately 10.5, 8, and 4.5 kb (Luneau *et al.*, 1991a; Weiser *et al.*, 1994), and KV3.2 probes hybridize to bands of approxi-

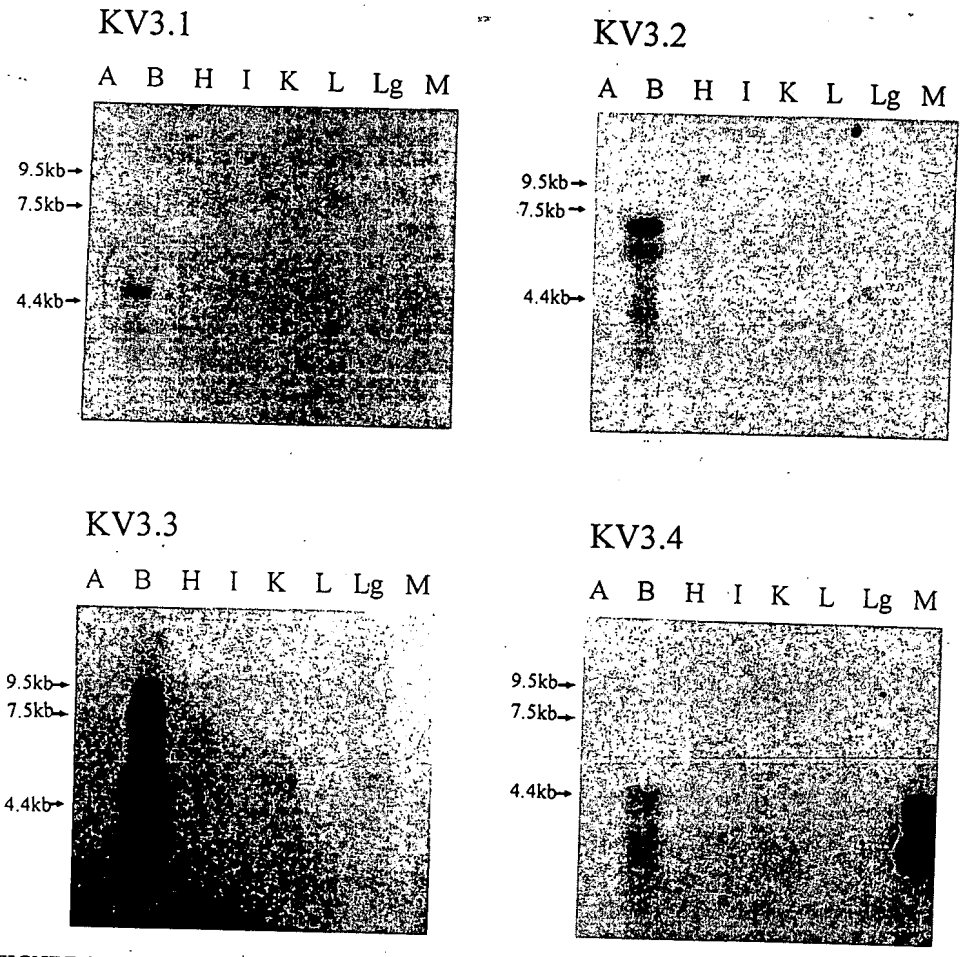


FIGURE 6 Tissue expression of four *Sh*III genes. Northern blot analysis of adult rat poly(A) RNA (3 μg per lane) from adrenal gland (A), brain (B), heart (H), intestine (I), kidney (K), liver (L), lung (Lg), and skeletal muscle (M) hybridized with probes specific to the KV3.1, KV3.2, KV3.3, and KV3.4 K^+ channel genes. The blots were exposed to X-ray film at −70°C with two intensifying screens for 2 days (KV3.1), 4 days (KV3.2, KV3.3), or 7 days (KV3.4). The positions of three RNA size markers (9.5, 7.5, and 4.4 kb) are indicated.

mately 7, 6, and 4 kb (Luneau *et al.*, 1991b; Rudy *et al.*, 1992; Weiser *et al.*, 1994). Interestingly, the 6-kb band is much weaker in thalamic RNA (Rudy *et al.*, 1992). KV3.3 probes hybridize to bands of approximately 8.5 and 5.5 kb (Vega-Saenz de Miera *et al.*, 1992; Weiser *et al.*, 1994) and KV3.4 probes to bands of 4.5 and 3.5 kb (Weiser *et al.*, 1994). Little data on the nature of the multiple transcripts of *Sh*III genes detected in Northern blots is currently available. For two of the members of this subfamily, KV3.2 (Luneau *et al.*, 1991b; Rudy *et al.*, 1992) and KV3.3 (Vega-Saenz de Miera *et al.*, 1992), probes specific to two 3′ coding alternatively spliced products produce banding patterns that are similar to each other. However, Luneau *et al.* (1991a) showed that for KV3.1, probes specific to KV3.1a hybridized to the 8-kb band whereas probes specific to the other alternatively spliced product, KV3.1b, hybridized to the 4.5-kb band. Nevertheless, both probes still hybridized to a relatively weak 10.5-kb band.

The meaning of the complex banding patterns just discussed remains unknown. Moreover, since the size of the coding regions of the cDNAs is less than 2.7 kb for all cases, the size of the transcripts seen in Northern blots is difficult to account for. The most obvious explanation for these phenomena would be incomplete splicing of the RNAs. However, for most (if not all) genes described to date, unspliced heterologous nuclear RNA is relatively short-lived and is certainly not the major RNA species, as would be the case for a number of these transcripts. Alternatively, these could be mature transcripts and the discrepancy in size from that of the coding regions could be the result of unusually large untranslated regions. The multiple bands could be explained by use of different polyadenylation sites and/or of as yet undefined alternative splice sites. Alternative splicing of 5′ UTRs is a possibility for which some evidence was discussed in the previous section.

The functional significance (if any) of these multiple transcripts from each *Sh*III gene is still unclear, but various possibilities exist. First, the mRNAs themselves may have a distinct subcellular localization. Such localization of mRNAs has been documented in the nervous system (Steward and Banker, 1992) and has been shown for some cases to depend on the 3′ untranslated region of the RNA. Second, 5′ untranslated regions have been shown in various systems (Kozak, 1992; Melefors and Hentze, 1993) to confer sequence-specific regulation of translation, which may indeed be the case for these transcripts. Yet another possibility is the regulation of mRNA stability (Saini *et al.*, 1990), a phenomenon that has been shown to depend on untranslated regions as well as length of the poly(A) tail. The preceding observations raise some interesting unanswered questions about the potential role of posttranscriptional regulation of *Sh*III K^+ channel gene expression.

D. *Electrophysiological Properties of* ShIII *Channels in* Xenopus *Oocytes*

Xenopus oocytes injected with *Sh*III transcripts express voltage-dependent K^+ currents that are absent in uninjected oocytes (Yokoyama *et al.*, 1989; T. McCormack *et al.*, 1990; Luneau *et al.*, 1991a,b; Rudy *et al.*, 1991b; Schröter *et al.*, 1991; Vega-Saenz de Miera *et al.*, 1992). The currents expressed by an alternatively spliced transcript of each of the four *Sh*III genes are shown in Fig. 7. The currents were recorded with a two-microelectrode voltage clamp that had sufficient time resolution to resolve the overall kinetic features of these currents.

A notable feature of the channels expressed by *Sh*III transcripts is their voltage dependence, which is nearly identical for all transcripts of this subfamily investigated to date (see Fig. 8A for representative conductance–voltage curves). Membrane potentials more positive than −10 mV are required to activate significant *Sh*III currents, a more depolarized value than that required to activate channels of the other subfamilies. The pharmacological properties of *Sh*III channels are also very similar. These channels are highly sensitive to both 4-aminopyridine (4-AP) and tetraethylammonium (TEA) and are not blocked by peptide toxins that block other *Sh* channels (see Table III).

*Sh*III currents do show variability in their kinetic properties. KV3.1 and KV3.2 transcripts express very similar slowly rising, delayed-rectifier type currents that inactivate extremely slowly. KV3.3 and KV3.4 transcripts, however, express A-type inactivating currents that are significantly different from each other. For instance, although macroscopic inactivation rates change with voltage in both cases, they are an order of magnitude faster in KV3.4 currents than in KV3.3 currents (Fig. 7). KV3.4 currents also rise faster than those carried by all other *Sh*III channels (Fig. 7).

KV3.3 and KV3.4 currents also differ in the voltage-dependence of steady-state inactivation. As a result of these differences, KV3.3 currents conduct in the steady state over a much broader window of potentials than KV3.4 currents (Fig. 8B). Vega-Saenz de Miera *et al.* (1992) suggested that the differences in the voltage dependence of steady-state inactivation of KV3.3 and KV3.4 currents could be explained by differences in the coupling between inactivation and channel opening, with KV3.4 channels inactivating

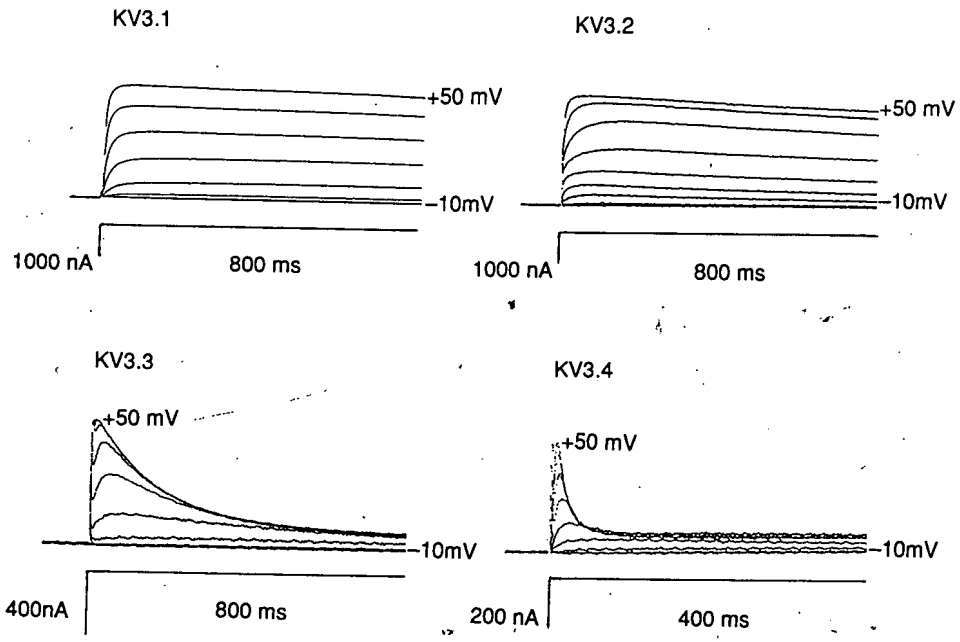


FIGURE 7 Currents expressed by one alternatively spliced variant of each of four *Sh*III genes in *Xenopus* oocytes. Shown are the currents recorded under voltage-clamp during depolarizing pulses from −60 to +50 mV in 10-mV increments delivered at a rate of one every 60 sec from a holding potential of −100 mV. Currents have been leak subtracted. The alternatively spliced variants used were KV3.1b, KV3.2a, KV3.3a, and KV3.4b. Note that the current increment decreases at high voltages. Modified with permission from Weiser *et al.* (1994).

from closed states much more readily than KV3.3 channels.

A provocative study by Ruppersberg *et al.* (1991a) showed that inactivated KV3.4a channels are able to conduct ions during repolarization. Apparently, these channels are unable to enter the resting closed state when the membrane is hyperpolarized until their inactivation is first removed, resulting in a long-lasting current (at voltages much more negative than those required for activation) while they are recovering from inactivation. KV3.3 channels, which inactivate more slowly, may or may not behave in a similar fashion. Such behavior could drastically affect the role played by these channels *in vivo* (see Section IV,F).

KV3.1a channels have also been expressed in human embryonic kidney cells (Critz *et al.*, 1993). Significantly, the overall properties of the currents are very similar to those seen in *Xenopus* oocytes, although a more detailed comparison of the KV3.1a currents in the two systems has not been reported. The electrophysiological and pharmacological properties of the channels formed by products of the four *Sh*III genes are summarized in Table III. Overall, *Sh*III channels display less functional variation than *Sh*I channels, which vary widely in voltage dependence, kinetics, and pharmacological properties (Stühmer *et al.*, 1989a).

Another aspect shared by all *Sh*III currents is a decline in conductance at membrane potentials more positive than +20 mV, although this feature is not quite as evident in KV3.1 currents (T. McCormack *et al.*, 1990; Rudy *et al.*, 1991b; Schröter *et al.*, 1991; Vega-Saenz de Miera *et al.*, 1992). In single-channel recordings, KV3.2a channels display a fast flickering at voltages more positive than +20 mV. This flickering produces a decline of the average *single-channel current* (Fig. 9) and is reminiscent of the effects of fast open-channel blockers on a number of channels (Hille, 1992). Based on observations such as those shown in Fig. 9, we proposed that the decline in conductance of *Sh*III channels results from fast voltage-dependent open-channel block by some intracellular component, perhaps Na^+ or Mg^{2+} (Rudy *et al.*, 1991b; Vega-Saenz de Miera *et al.*, 1992). These ions have been shown to produce similar block of other voltage-gated K^+ channels, but at more positive potentials (Linsdell *et al.*, 1990; Lopatin and Nichols, 1993). Rettig *et al.* (1992) showed that a similar blockage of channels expressed by a KV3.4 construct lacking the N-terminal 28 residues is abolished in inside-out patches on removal of Mg^{2+} from the bath solution.

The significance of so many different alternatively spliced transcripts from *Sh*III genes is quite baffling, since none of the alternatively spliced transcripts

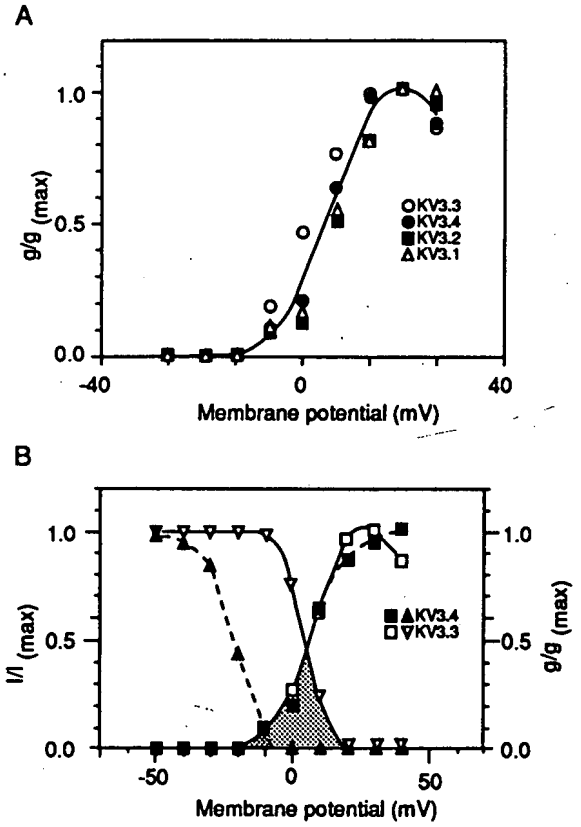


FIGURE 8 (A) Conductance–voltage relationship of four *Sh*III currents. Plots of average normalized conductance (g/g_{max}; conductance at the indicated potential divided by the maximum conductance) as a function of voltage. A single curve through the data of the four channels was drawn by hand. (B) Comparison of the conductance–voltage and steady-state inactivation–voltage relationships of KV3.3 and KV3.4 currents. Plots of normalized conductance (g/g_{max}; squares) and steady-state inactivation (I/I_{max}; triangles) as a function of voltage. Note that, in the steady state, KV3.3 channels conduct over a much broader window of potentials than KV3.4 channels (see area filled with dots and bars, respectively).

tested to date (KV3.1a, Yokoyama *et al.*, 1989; KV3.1b, Luneau *et al.*, 1991a; KV3.2a, T McCormack *et al.*, 1990; KV3.2b, Luneau *et al.*, 1991a; KV3.2d, H. Moreno and B. Rudy, unpublished observations; KV3.4a, Schroter *et al.*, 1991; KV3.4b, Rudy *et al.*, 1991b) expresses currents in *Xenopus* oocytes that are noticeably different from those of any other transcript from the same gene. However, more in-depth analysis of the channels expressed by these transcripts may reveal previously overlooked subtle differences. Alternatively, these channels may respond differently to various second messenger systems (see Section IV,G). Alternative splicing may thus allow for isoform-specific differences in the modulation of channel function by neurotransmitter and neuropeptide-induced cellular responses. The divergent C termini could be used as well in protein–protein interactions, thus conferring isoform-specific channel localization, mobility, and other forms of modulation.

E. Functional Consequences of Heteromultimer Formation between ShIII *Proteins*

Formation of heteromultimeric channels results in a large increase in the potential number of different channels that a given set of *Sh* subunits could form. This property leads to the formation of channels with subtle, but often physiologically significant, differences (e.g., Po *et al.*, 1993). For example, if 10 *Sh*III subunits are capable of forming heteromultimeric channels, assuming the channels are tetramers 715 channels with differing subunit composition could be formed.[2] Most studies investigating heteromultimer formation have used experiments in which different pairs of cRNAs are co-injected in *Xenopus* oocytes or coexpressed in other cell types. Heteromultimer formation is suggested by the fact that, when two different subunits are coexpressed, currents are obtained that cannot be the algebraic sum of the currents expressed by each channel independently.

In general, heteromultimeric *Sh*I channels have properties that are intermediate between those of the corresponding homomultimers, although some properties might be closer to those of one or the other homomultimer (Isacoff *et al.*, 1990; K. McCormack *et al.*, 1990; Ruppersberg *et al.*, 1990; MacKinnon *et al.*, 1991). Since all *Sh*III channels have similar voltage dependencies, this parameter was expected not to change in heteromultimers. This expectation has been confirmed experimentally; when two different *Sh*III cRNAs are injected into oocytes, the total current has a voltage dependence similar to that of the *Sh*III homomultimers (H. Moreno, E. Vega-Saenz de Miera, and B. Rudy, unpbulished observations). Moreover, and perhaps not surprisingly, the currents recorded in oocytes co-injected with KV3.1 and KV3.2 cRNAs, which produce similar currents in a homomultimer channel, are similar to the currents of KV3.1 or KV3.2 alone.

Oocytes co-injected with *Sh*III cRNAs that express currents that inactivate at different rates when injected alone expressed currents suggestive of heteromultimer formation (Weiser *et al.*, 1994). One of the most interesting results from these co-injection experiments is the observed changes when KV3.4 cRNA is co-injected with KV3.1 cRNA. These oocytes had fast inactivating currents that were several-fold larger than those seen in oocytes injected with the same amount of KV3.4 cRNA alone (Fig. 10). This result is contrary

[2] The number of different subunit combinations can be calculated from the equation $[p + (n - 1)]!/[p!(n - 1)!]$, where p is the aggregation number and n is the number of subunits.

TABLE III **Electrophysiological and Pharmacological Properties of Mammalian *Sh*III Channels in *Xenopus* Oocytes**[a,b]

	Activation			Inactivation				Pharmacology—IC_{50} (m*M*)					
Gene	$V_{.5}$ (mV)	k (mV)	Δt (msec)	$V_{.5}$ (mV)	k (mV)	τ (msec)	g (pS)	TEA	4AP	Quinine	DTX	CTX	MCDP
KV3.1	+10	8	24	—	—	—	16	0.150	0.6	1	NB	NB	NB
KV3.2	+10	7	23	—	—	—	16	0.150	0.9	ND	NB	NB	NB
KV3.3	+7	6	25	+5.2	6.1	240	14	0.140	1.2	ND	ND	ND	ND
KV3.4	+14	8.5	9	−20	7.2	20	12	0.200	0.6	0.5	NB	ND	NB

[a] Data derived from Yokoyama *et al.* (1989), T. McCormack *et al.* (1990), Rudy *et al.* (1991b), Rettig *et al.* (1992), Vega-Saenz de Miera *et al.* (1992), and H. Moreno and B. Rudy (unpublished observations).

[b] Abbreviations: For activation: $V_{.5}$, membrane potential at which the conductance is half maximal; k, slope of normalized g/V curve; Δt, time for the current to rise to 90% of its final value for noninactivating currents and time to peak for inactivating currents, in both cases at +40 mV. For inactivation: $V_{.5}$, membrane potential of a 1-sec (for KV3.4) and 2-sec (for KV3.3) prepulse producing 50% inactivation; k, slope of the steady-state inactivation vs. voltage curve; τ, time constant of inactivation at +30 mV. g, single channel conductance in physiological K^+ concentrations, in cell-attached patches. For pharmacology: IC_{50} (m*M*), concentrations blocking 50% of the current. TEA, tetraethylammonium; 4AP, 4-aminopyridine; DTX, dentrotoxin; CTX, charybdotoxin; MCDP, mast cell degranulating peptide; NB, no block; ND, not determined.

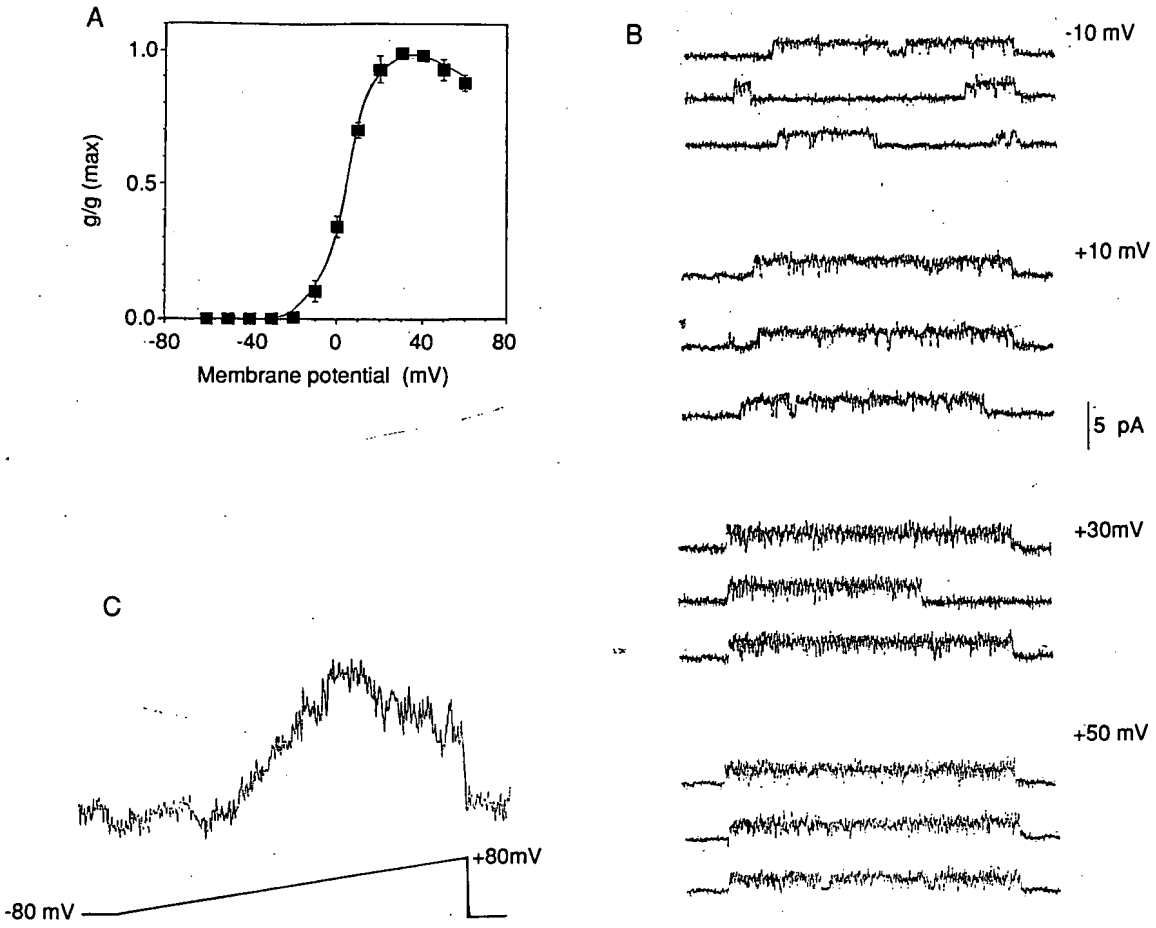


FIGURE 9 Voltage-dependent block of KV3.2a channels. (A) Normalized conductance–voltage curve of KV3.2a channels obtained from macroscopic currents in *Xenopus* oocytes. (B) Representative single KV3.2a channel records at four different voltages in a cell-attached patch on an oocyte injected with KV3.2a cRNA. (C) Ensemble average of 32 sweeps obtained from the same patch used in B during a voltage ramp from −80 to +80 mV.

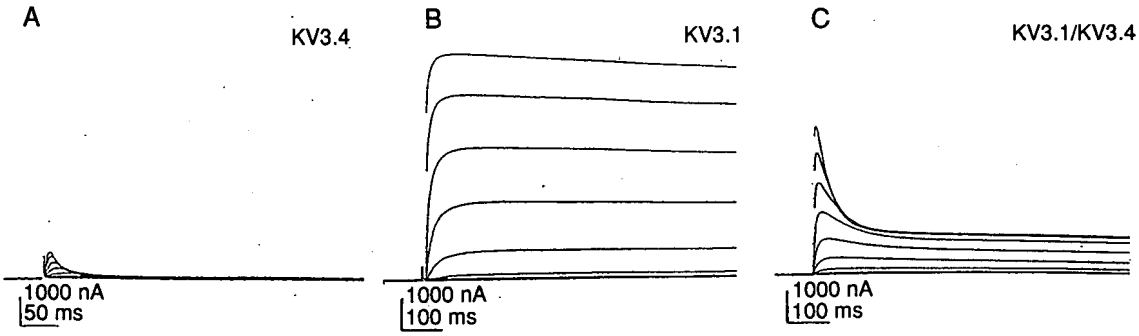


FIGURE 10 KV3.1–KV3.4 heteromultimeric channels in *Xenopus* oocytes. (A) Currents recorded in a representative oocyte injected with KV3.4b cRNA alone. (B) Currents recorded in a representative oocyte injected with KV3.1b cRNA alone. (C) Currents recorded in a representative oocyte injected with the same amount of KV3.4b cRNA as the oocyte in A plus the same amount of KV3.1b cRNA as the oocyte in B. The currents shown here are representative of several similar experiments. For example, the average transient current at +40 mV of 8 oocytes injected with KV3.4b cRNA alone was 300 nA; that in oocytes co-injected with KV3.1b and KV3.4b was 1800 nA. Modified with permission from Weiser *et al.* (1994).

to what might be expected from the algebraic sum of two independent currents and is consistent with the formation of heteromultimeric channels containing one or more KV3.4 subunits and the remaining KV3.1 subunits (Weiser *et al.*, 1994). The amplification of the currents is caused by both an increase in single channel conductance in the heteromultimeric channels (compared with that of KV3.4 channels) and the fact that apparently less than four KV3.4 subunits are sufficient to impart fast inactivating properties on the channel. Similar studies with *Sh*I subunits have also shown that the presence of a single inactivating subunit is sufficient to impart inactivating properties on the resultant channels (e.g., Isacoff *et al.*, 1990; K. McCormack *et al.*, 1990; Ruppersberg *et al.*, 1990; MacKinnon *et al.*, 1991). This behavior can be understood in terms of the N-terminal "ball and chain" model of inactivation (Hoshi *et al.*, 1990; Zagotta *et al.*, 1990; see Section IV,G). The "ball and chain" of a single inactivating subunit is sufficient to inactivate the channel, although the rate of inactivation increases with the number of inactivating subunits (MacKinnon *et al.*, 1991), as was also the case for KV3.1–KV3.4 heteromultimers (Weiser *et al.*, 1994). Similar results have been obtained in oocytes coinjected with KV3.4 and KV3.2 or KV3.3 (H. Moreno, unpublished observations).

F. Role of ShIII *Channels in Excitability*

Voltage-dependent K^+ currents modulate various important aspects of neuronal excitability including membrane resting potential, action potential waveform, firing patterns, neurotransmitter release, and postsynaptic potentials. The extent of the involvement of a given K^+ channel in these processes is dependent on the number of channels and their conductance, voltage dependence, kinetics, and subcellular localization, as well as on the balance of other currents (Hille, 1992). Further, these properties are often altered by cellular responses to neurotransmitters and neuropeptides, making K^+ channels important mediators of the longer term effects of synaptic transmission (Levitan, 1988; Kaczmareck and Levitan, 1987; Rudy, 1988).

As discussed in the preceding sections, transcripts from the four *Sh*III genes express K^+ channels in *Xenopus* oocytes that start activating at highly depolarized potentials. Heteromultimer formation allows for the existence of channels with this characteristic possessing a wide range of inactivation kinetics. Particularly interesting is the finding that small numbers of KV3.4 subunits might be sufficient to impart fast inactivating properties on channels composed mainly of other *Sh*III subunits (see Section IV,E). Assuming that native *Sh*III channels have properties similar to those seen in oocytes, certain predictions can be made about the kinds of electrical activity native *Sh*III channels may or may not be involved in, and what their effects might be on neuronal excitability.

Voltage-gated K^+ channels can affect fast Na^+ action potential waveforms primarily by their influence on the repolarization of the spike. *Sh*III channels could easily be active at the depolarized potentials (+10 to +30 mV) characteristic of these spikes. In fact, Perney *et al.* (1992) suggested that the physiological role of KV3.1 channels is to shorten the duration of action potentials in fast-firing neurons. In support of their hypothesis, these investigators reported studies with AtT20 cells transfected with the *ras* oncogene, which caused a 20-fold reduction in the width of action potentials relative to untransfected cells and was associated with the induction of KV3.1 mRNA (Hemmick *et al.*, 1992).

Although it is conceivable that KV3.1 channels (or KV3.2 channels, which express similar currents) act to shorten the duration of action potentials in fast-firing neurons, this hypothetical function must be reconciled with the relatively slow activation kinetics of these channels (see Section IV,D) and the short duration of neuronal fast action potentials (<1 msec at 35–37°C). Of course, channel density could make up for the small percentage of these channels that would be activated during a spike and would provide sufficient current to repolarize the membrane. Alternatively, KV3.1 or KV3.2 channels could activate much faster during an action potential as a result of having been exposed to depolarized voltages below those necessary to produce measurable current. KV3.1 and KV3.2 channels could easily be activated, however, during action potentials of longer duration.

The spike frequency of trains of action potentials depends mainly on the membrane potential and resistance between spikes. These parameters are governed in large part by K^+ channels that are activated at voltages below the threshold of action potentials. However, the voltage dependence of *Sh*III channels precludes their involvement in subthreshold phenomena such as regulation of spike frequency or latency to first spike. In this context, the work of Ruppersberg *et al.* (1991a), described in Section IV,D, provides an interesting caveat to this generalization. If inactivated KV3.4 channels are indeed able to conduct ions at negative potentials while they recover from inactivation, they could have quite interesting effects on firing patterns. Specifically, the first spike in a train could open and then quickly inactivate these channels. On repolarization of the membrane, the current through

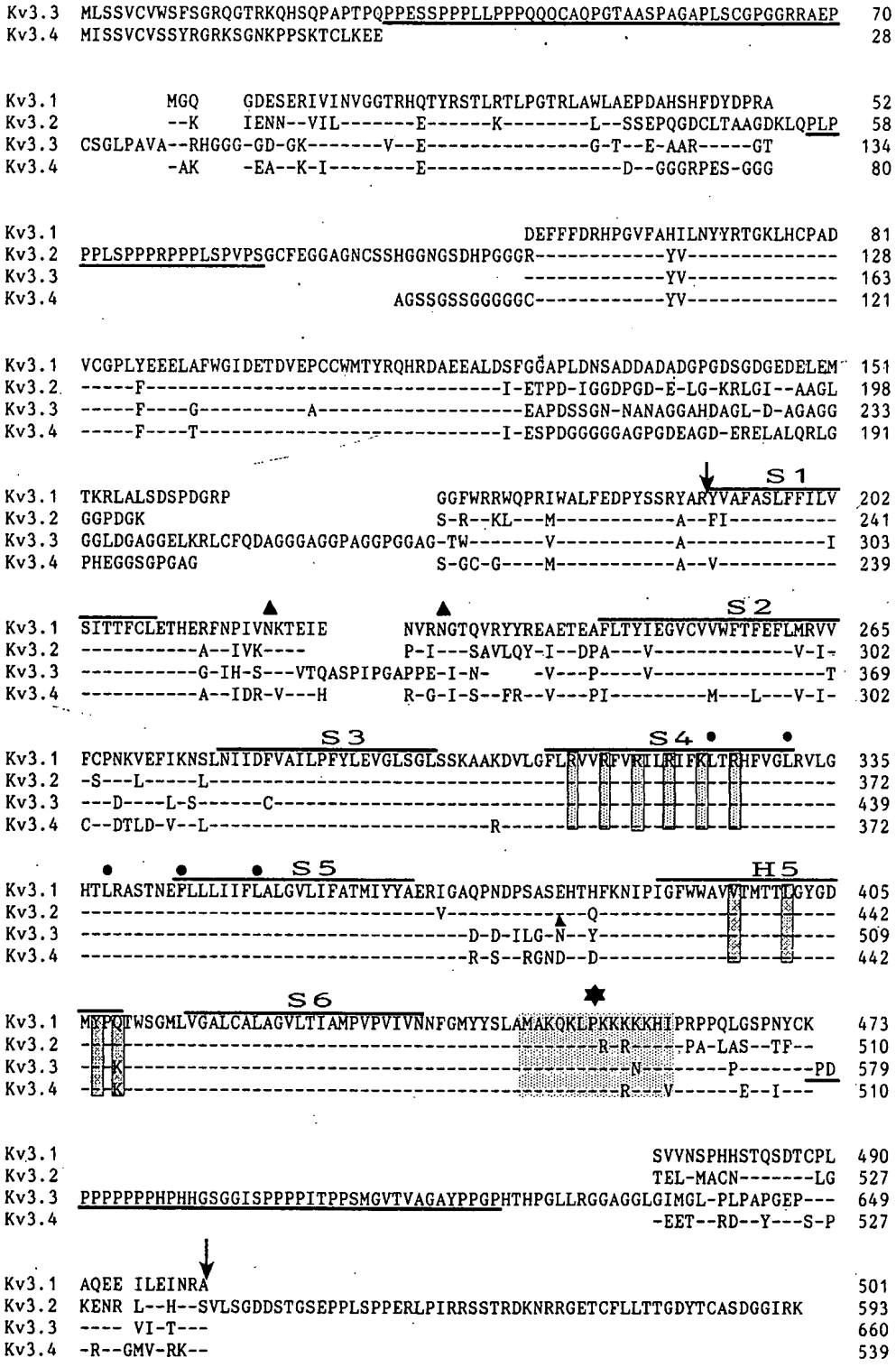


FIGURE 11 Comparison of the constant region of K^+ channel proteins encoded by four *Sh*III genes. Predicted amino acid sequences of the constant region (the sequences preceding the alternatively spliced carboxyl ends) of proteins encoded by *Sh*III transcripts. The sequences are aligned to maximize long stretches of identity. Amino acids identical to those in KV3.1 are shown with a dash. Gaps required for optimal alignment are shown as blanks. Note that the second methionine of KV3.3 and KV3.4 has been aligned with the starting methionines of KV3.1 and KV3.2. The sequences between the first and second methionines of KV3.3 and KV3.4 are important for channel inactivation. The first arrow marks the end of the sequences encoded by exon 1 and the second arrow marks the end of the sequences encoded by exon 2. The S1–S6 and H5 domains are overlined. Proline-rich sequences in KV3.2 and KV3.3 are underlined. Two putative *N*-glycosylation sites in all *Sh*III proteins in the linker between the S1 and S2 domains are indicated with triangles. KV3.3 has an additional putative *N*-glycosylation site in the S5–H5 linker (also indicated with a triangle). The positively charged residues in S4 have been

KV3.4 channels recovering from inactivation could prevent the next spike or delay its onset.

Both the pre- and the postsynaptic sides of synaptic transmission can be modulated by K^+ channels. The amount of neurotransmitter release from the presynaptic terminal is dependent on the amount of Ca^{2+} entry through voltage-gated Ca^{2+} channels. Activation of K^+ channels in the presynaptic terminal can limit the magnitude or duration of local depolarization and, thus, Ca^{2+} entry and transmitter release. On the other side of the synapse, activation of K^+ channels could limit the spread of the depolarization caused by receptor activation. *Sh*III channels could play a role in both of these processes, provided that presynaptic potentials or excitatory postsynaptic potentials (EPSPs) reach voltages sufficient to activate these channels. This event would be more likely in areas devoid of large numbers of K^+ channels that activate at lower voltages.

The vagueness of the preceding discussion underscores the importance of studies of *Sh*III channels that are aimed at elucidating the electrophysiological properties and subcellular localization of K^+ channels containing *Sh*III subunits *in vivo*. The state of present work on these problems and future directions for the field are discussed in later sections of this chapter.

G. *Structural and Functional Domains in* ShIII *Proteins*

The overall structure of mammalian *Shaw*-related or *Sh*III proteins (Yokoyama *et al.*, 1989; T. McCormack *et al.*, 1990,1991; Luneau *et al.*, 1991a,b; Rudy *et al.*, 1991b,1992; Schröter *et al.*, 1991; Vega-Saenz de Miera *et al.*, 1992; Ried *et al.*, 1993) is similar to that of all other *Sh* proteins (reviewed by Jan and Jan, 1990a;1992; Perney and Kaczmareck, 1991; Rudy *et al.*, 1991a; Pongs, 1992) and other channel proteins of the S4 superfamily (see Fig. 1). A comparison of the constant region (prior to divergence of the carboxyl ends) of the protein products of the four *Sh*III genes is shown in Fig. 11. *Sh* proteins are several hundred amino acids in length and are characterized by a core membrane region consisting of six putative membrane-spanning domains designated S1, S2, S3, S4, S5, and S6 flanked by intracellular amino and carboxyl domains of variable lengths (see Figs. 3, 11). Domains S1–S3 and S5–S6 are hydrophobic and are thought to span the membrane as α helices. The S4 domain is amphipathic, but is also believed to be membrane spanning. The topology of the S1–S6 domains is not known, but models have been proposed based on the idea that less conserved, highly hydrophobic domains are likely to interact with lipid whereas highly conserved domains may be involved in protein–protein interactions and may be located in the interior of the protein (Guy, 1989). In addition to having a similar overall structure, *Sh*III proteins and *Sh* proteins of the other subfamilies share significant amino acid sequence similarity (~40% sequence identity in a core region that includes all the membrane portion of the polypeptides). Details of the structure of *Sh* proteins that are not addressed here can be found in several reviews (Jan and Jan, 1990a;1992; McKinnon, 1991; Miller, 1991; Rudy *et al.*, 1991a; Pongs, 1992).

As is the case for *Sh* proteins of the other subfamilies, *Sh*III proteins are most similar in the core membrane region (which corresponds roughly to the sequence encoded in exon 2 of *Sh*III genes; see Section IV,B). In this region, *Sh*III proteins share ~85% amino acid identity, compared with ~70% *overall* amino acid identity, indicating that the regions preceding and following the core region are less conserved (see Figs. 3, 11). Most of the differences among *Sh*III proteins are localized to parts of the amino end region preceding the first membrane-spanning domain, the carboxyl domain following the membrane portion of the polypeptide, and the S1–S2 and S2–S3 linkers.

The conservation of the core region among *Sh*III proteins is somewhat higher than that among different *Sh*I proteins (75–80% identity). This conservation may explain why the channels composed of *Sh*III proteins display less functional variability than those composed of *Sh*I proteins (see Section III,D). The core regions of *Sh*II proteins are even more conserved than those of *Sh*III proteins (Frech *et al.*, 1989; Hwang *et al.*, 1992). *Sh* proteins also share several structural and functional domains, the most characteristic being the H5 or pore domain and the S4 or voltage-sensor domain.

1. H5 or Pore (P) Domain

Between the fifth and the sixth membrane-spanning domains is a highly conserved and somewhat hydrophobic sequence knows as H5 (or SS1–SS2 by some authors; Jan and Jan, 1990a; Perney and Kaczmareck, 1990; Rudy *et al.*, 1991a; Pongs, 1992; see also Chapter 2). H5 domains are present not only

boxed. The leucines in the *Sh* leucine heptad repeat adjacent to the S4 domain are indicated with a filled circle. The fourth residue in the repeat is phenylalanine in *Sh*III proteins, but leucine in all other *Sh* proteins. Residues in the H5 domain that were found by mutagenesis to be important in determining pore properties of KV3.1 are boxed. A cluster of positively charged residues after the S6 domain is indicated with a star.

in the K^+ channels of the S4 superfamily (the *Sh, eag,* and *slo* families) but also in inward rectifier channels that otherwise bear no resemblance to the S4-containing K^+ channels (Ho *et al.*, 1993; Kubo *et al.*, 1993). The only known exception is the I_{SK} channel, which lacks a clear H5 domain. Mutations of the H5 domain in *Drosophila* and mammalian *Sh* proteins affect ion selectivity, open-channel conductance, and blockade by TEA, providing strong evidence that this domain contributes to the formation of the channel pore (MacKinnon and Yellen, 1990; Yellen *et al.*, 1991; Yool and Schwarz, 1991; Hartmann *et al.*, 1991; Kirsch *et al.*, 1992a,b). In one of these experiments (Hartmann *et al.*, 1991; Kirsch *et al.*, 1992a,b), the H5 domain of KV2.1 (a mammalian *Shab*-related protein) was replaced with the H5 domain from a mammalian *Sh*III protein (KV3.1), yielding a chimeric protein expressing channels with permeability properties characteristic of KV3.1 channels, including single-channel conductance and internal TEA sensitivity. Na^+ and Ca^{2+} channels contain a domain in an analogous position (but with a different amino acid sequence), known as SS1–SS2, that participates in the regulation of ion permeability in these channels (Guy and Conti, 1990; Heinemann *et al.*, 1992).

In the accepted topology of *Sh* proteins, the H5 domain begins at the extracellular side of the membrane. The H5 domain of each subunit has been proposed to form an antiparallel β hairpin loop that enters and exits the membrane so the pore is a hole in the middle of an eight-stranded β barrel (Guy, 1989; Guy and Conti, 1990). Consistent with this hypothesis, mutagenesis studies demonstrate that the H5 domain is accessible from both sides of the membrane. Mutations of amino acid residues near the beginning and the end of the H5 domain affect channel block by external TEA, whereas mutations of residues near the middle of H5 affect mainly blockade by internal TEA (Yellen *et al.*, 1991).

Residue 19 in the H5 domain of all mammalian *Sh*III proteins is a tyrosine (Fig. 11). In *Drosophila Shaker* channels, a tyrosine in this position conferred high sensitivity to external TEA (MacKinnon and Yellen, 1990). All *Sh*III channels are blocked by submillimolar concentrations of external TEA (see Table III). Tyr 19 probably contributes to the sensitivity of *Sh*III channels to external TEA, but is clearly not sufficient since KV2.1 channels have a tyrosine in this position but are about 20-fold less sensitive to external TEA than *Sh*III channels. Moreover, the KV2.1 chimera with a KV3.1 H5 domain described earlier (KV2.1–KV3.1 chimera) was less sensitive to external TEA than KV3.1 channels (Hartmann *et al.*, 1991).

Mutagenesis studies, particularly those with the KV2.1–KV3.1 chimera (Hartmann *et al.*, 1991; Kirsch *et al.*, 1992a,b), have been useful in identifying other residues that contribute to the pore properties of *Sh*III subunits. These residues include a leucine and a valine in the middle of the KV3.1 H5 domain that are replaced by valine and isoleucine, respectively, in KV2.1 (Fig. 12A). These residues have been shown to influence the K^+ conductance, mean open time, and relative K^+/Rb^+ conductance of KV3.1 channels. These residues are also important in determining the sensitivity of KV3.1 channels to internal TEA blockade, which is significantly lower in these channels (Taglialatela *et al.*, 1991; Kirsch *et al.*, 1992a,b). KV3.2 proteins are identical to KV3.1 in the region of the H5 domain; however, KV3.3 and KV3.4 have a lysine two positions C-terminal to Tyr 19, whereas KV3.1 and KV3.2 have a glutamine at this position. Substitution of this glutamine for lysine in the KV2.1–KV3.1 chimera, a change resulting in an increase in the charge close to the putative external mouth of the channel, reduced the single-channel conductance to a degree similar to the difference in single-channel conductance between KV3.1 (or KV3.2) and KV3.3 or KV3.4 channels (see Table III). This substitution also reduced the external TEA sensitivity 5-fold; however, KV3.3 and KV3.4 channels have external TEA sensitivities that are not very different from those of KV3.1 and KV3.2 (see Table III). Other residues, or the structure of the pore itself, may explain these differences. Indeed, a triple mutation was required (V8I, L13V, Q21K) to restore the external TEA sensitivity of the KV2.1–KV3.1 chimera to that of KV2.1 channels (Kirsch *et al.*, 1992a,b).

Peculiar to mammalian *Sh*III proteins is the presence of a 9-residue insert just prior to the beginning of the H5 domain that results in elongation of the linker between the S5 and the H5 domains relative to all other *Sh* channels (Fig. 12A). *Sh*III proteins expressing inactivating and noninactivating channels differ in the sequence of this insert (Fig. 11). The insert is nearly identical in KV3.1 and KV3.2 proteins that express delayed-rectifier type currents, but different in KV3.3 and KV3.4 that express inactivating channels.

2. S4 Domain

S4 domains, located between the third (S3) and the fourth (S5) hydrophobic segments, are characterized by the repetition of a motif consisting of two neutral residues (usually hydrophobic, except toward the carboxyl end of S4) and one positively charged residue. In *Sh* proteins, the positively charged residue is usually arginine, except toward the carboxyl end of S4 where some lysines are located. S4 domains are also present in a number of other voltage-gated channels including K^+ channels of the *eag* family, Na^+ and Ca^{2+} channels,

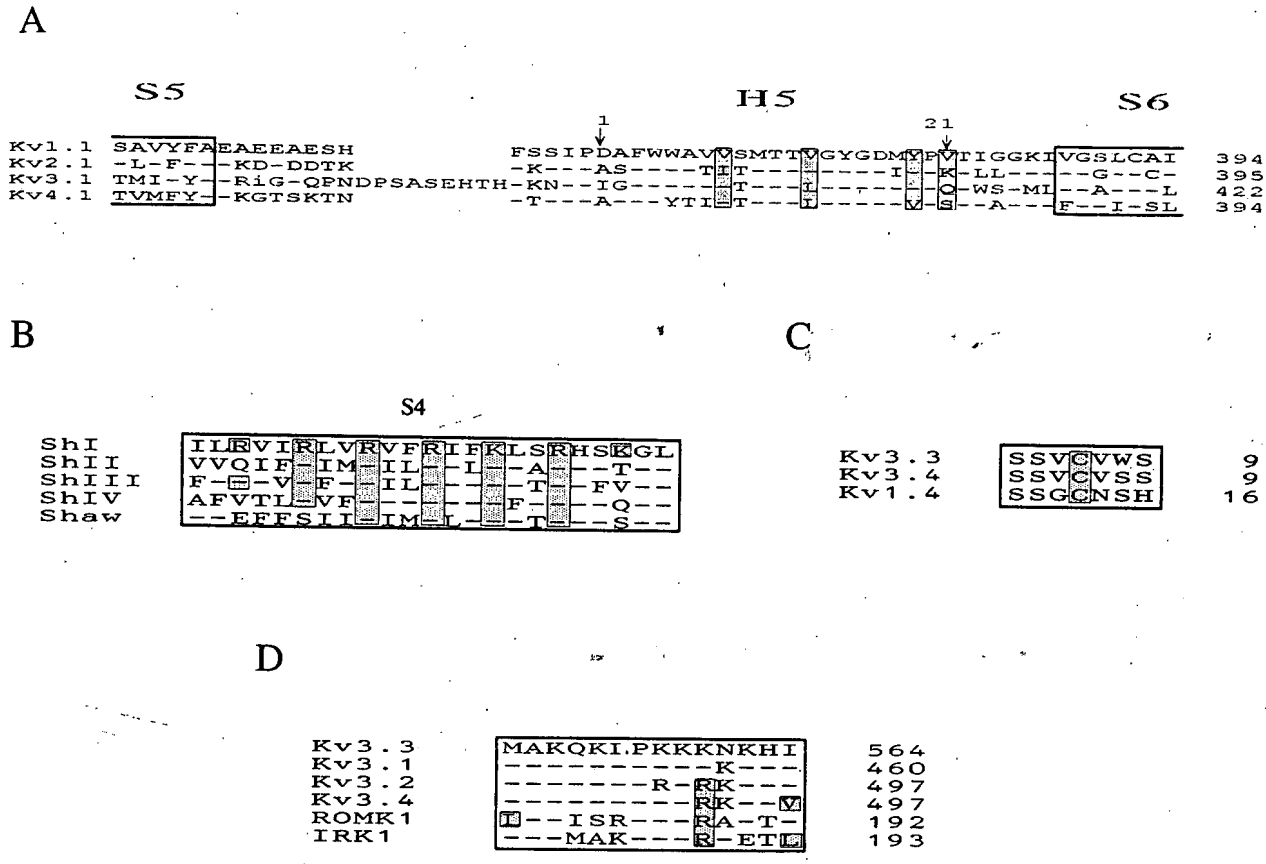


FIGURE 12 (A) Comparison of the sequence between the S5 and S6 domains (including the H5 domain) of mammalian *Sh* proteins of different subfamilies. Note the 9-residue insert in KV3.1. Other *Sh*III proteins have a similar insert (see Fig. 11). The sequences are aligned to maximize long stretches of identity. Gaps required for optimal alignment are shown as blanks. The amino acids in the H5 region have been arbitrarily numbered from 1 to 21. Residues in the H5 domain that were found by mutagenesis to be important in determining pore properties of KV3.1 are boxed (see text). (B) Comparison of the sequence of the S4 domains of mammalian *Sh* proteins and the *Drosophila Shaw* protein. Positively charged residues have been boxed. (C) Conserved sequence containing a cysteine residue at the amino end of KV3.3, KV3.4, and KV1.4 proteins. (D) A region rich in positively charged residues that follows the S6 domain of the protein products of the four *Sh*III genes is compared with a similar sequence in two inward rectifier channels. In A, C, and D the numbers on the right represent the position of the last residue shown relative to the starting methionine. In A, B, and D a dash represents an amino acid identical to that of the sequence shown in the top row.

and some channels that are thought not to be primarily voltage gated, such as cyclic-nucleotide-activated channels (Kaupp *et al.*, 1989; Dhallan *et al.*, 1990; Jan and Jan, 1990b) and Ca^{2+}-activated K^+ channels of the *slo* family (Atkinson *et al.*, 1991). The number of repetitions of the sequence motif X–X–R/K, where X is a hydrophobic or neutral residue, in the S4 domain is characteristic of the proteins of each *Sh* subfamily: seven in *Sh*I proteins, five in *Sh*II proteins, six in mammalian *Sh*III proteins, and five in *Sh*IV proteins (Fig. 12B). Curiously, the *Drosophila Shaw* protein contains a distorted S4 domain containing four positively charged residues, one negatively charged residue, and one serine instead of one of the arginines (Fig. 12B).

Mutagenesis experiments on Na^+ and *Sh* K^+ channels support the notion that the S4 domain acts as a voltage sensor (Noda *et al.*, 1986; Stühmer *et al.*, 1989b; Liman *et al.*, 1991; Papazian *et al.*, 1991; see also Chapter 3). However, the mechanism of voltage-dependent gating and the role of the S4 domain in this mechanism is far from understood and might be quite complex, involving structures outside the S4 domain (Liman *et al.*, 1991; K. McCormack *et al.*, 1991). For example, clearly not all positive charges are equivalent; in addition, for some positions, the nature of the positively charged residue (arginine or lysine) is important. Considering these results, it is not surprising that no clear-cut correlation exists between the reported slope of the conductance–voltage relationship (gating charge)

of *Sh* channels and the number of positive charges in the S4 domain. Nevertheless, the fact that the structure of the S4 domains is constant for each subfamily is intriguing.

Other potentially important domains of *Sh*III proteins may or may not be found in *Sh* proteins of other subfamilies, or even in all *Sh*III proteins. Starting at the amino end, several of these domains are described in the following sections.

3. Amino End "Ball and Chain" Domains in Inactivating *Sh*III Proteins

KV3.3 and KV3.4 proteins, which express inactivating channels, differ from those that do not inactivate by an N-terminal insert of 78 residues in KV3.3 and 28 residues in KV3.4 (see Fig. 11). At the C-terminal end of these inserts is a methionine that can be aligned with the starting methionine of KV3.1 and KV3.2 to produce proteins with similar sequence (Fig. 11).

In *Drosophila Shaker* channels, the N termini are involved in a fast inactivation process known as N-inactivation. In addition, another inactivation process known as C-inactivation is dependent on structures toward the C terminus of the protein (perhaps between H5 and S6; Hoshi *et al.*, 1991). Deletions of the N terminus in *Shaker* channels results in removal of the N-inactivation (Hoshi *et al.*, 1990; Zagotta *et al.*, 1990). The N terminus is hypothesized to act as a "ball and chain" (Hoshi *et al.*, 1990; Zagotta *et al.*, 1990). The tethered "ball" is proposed to produce inactivation by occluding the pore of open channels (Armstrong and Bezanilla, 1977; Hoshi *et al.*, 1990; Zagotta *et al.*, 1990).

The presence of clear N-terminal inserts in inactivating KV3.3 and KV3.4 channels suggests that these channels also inactivate by a "ball and chain" mechanism. In support of this hypothesis, deletion of the 78 residues of the N-terminal insert of KV3.3 and the 28 residues of the N-terminal insert of KV3.4 results in the expression of channels that do not inactivate (Fig. 13; see also Rettig *et al.*, 1992). Whether the differences in inactivation properties of the channels formed by KV3.3 and KV3.4 proteins (Section IV,D) are the results of the differences in their amino ends still remains to be determined.

4. A Domain for the Regulation of Channel Activity by the Redox Potential

The inactivating inserts of KV3.3 and KV3.4 contain a cysteine residue surrounded by a very conserved sequence (Fig. 12C). A very similar sequence is found

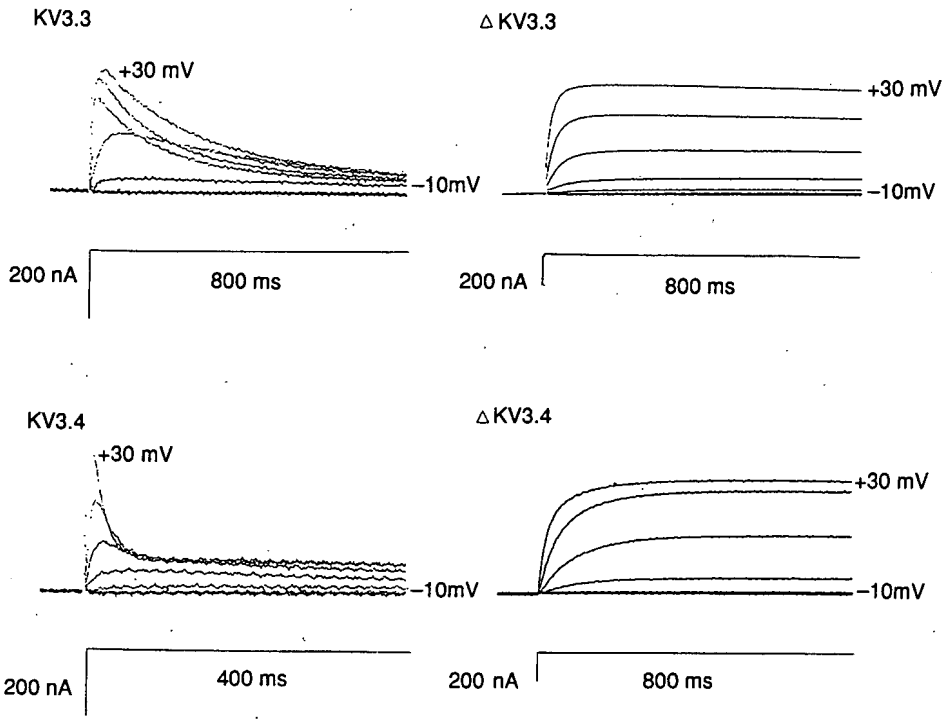


FIGURE 13 Deletion of the N-terminal inserts of KV3.3 and KV3.4 removes inactivation. ΔKV3.3 and ΔKV3.4 correspond to constructs in which the first 78 amino acids of KV3.3a and the first 28 amino acids of KV3.4b, respectively, have been deleted. Shown are the currents recorded under voltage clamp during depolarizing pulses from −60 to +30 mV in 10-mV increments, delivered at a rate of one every 60 sec from a holding potential of −100 mV. Currents have been leak subtracted.

in KV1.4, a fast-inactivating *Shaker*-related mammalian channel (Fig. 12C). The conservation of this sequence is even more notable since otherwise very little amino acid sequence conservation is seen in the N-termini of these proteins (Stühmer *et al.*, 1989a; Rudy *et al.*, 1991b; Schroter *et al.*, 1991; Vega-Saenz de Miera *et al.*, 1992).

Ruppersberg *et al.* (1991b) showed that exposure of the cytoplasmic surface of KV1.4 or KV3.4 channels to air, presumably to oxygen (in inside-out excised membrane patches), resulted in removal of the inactivation process. In contrast to the effects of N-terminal deletions and other treatments, this inactivation removal was reversible. Inactivation was restored if the patch was reinserted into the cell or by exposure to reduced glutathione or dithiothreitol (DTT; Ruppersberg *et al.*, 1991b). The effects of oxidation were not seen in channels expressed from a KV1.4 protein in which the cysteine was replaced with serine (Ruppersberg *et al.*, 1991b). A model to explain how the redox state of this cysteine may modulate channel inactivation was proposed by these investigators.

Similarly, exposure of oocytes expressing KV1.4, KV3.3, or KV3.4 channels to high micromolar concentrations of external H_2O_2 resulted in a reversible removal of inactivation (Vega-Saenz de Miera and Rudy, 1992). Concomitant with inactivation removal there was a significant increase in current magnitude that was particularly large in KV3.4 injected oocytes. The effects of H_2O_2 were specific to these three channels. No effect was detected in oocytes expressing channels that lack the conserved cysteine-containing sequence, including noninactivating KV3.2 channels, inactivating *Drosophila Shaker* channels (Vega-Saenz de Miera and Rudy, 1992), and inactivating KV4.1 and KV4.2 channels (of the *Sh*IV subfamily) (P. Serodio and B. Rudy, unpublished observations). Effects similar to those of H_2O_2 were seen on exposure to *N*-ethyl maleimide, a reagent that alkylates free sulfhydryls; in this case the effects were, as expected, irreversible.

5. Proline-Rich Domains

KV3.2 and KV3.3 proteins have proline-rich areas (underlined in Fig. 11) in regions of the protein that are thought to be cytoplasmic. One such sequence is found in KV3.2 in the N-terminal side of the S1 domain, and one is found in the C-terminal region of KV3.3 after the S6 domain. In addition, the inactivating N-terminal insert of KV3.3 is also proline rich.

T. McCormack *et al.* (1990) first observed one such proline-rich insert in KV3.2a that had the sequence PPLSPPPRPPPLSP. These investigators suggested that the serines in this sequence might be sites of cytoplasmic O-glycosylation, based on similarities with the sequences surrounding serines in other proteins that are known to undergo cytoplasmic O-glycosylation.

More recently, researchers have found that proline-rich sequences such as those seen in KV3.2 and KV3.3 are binding sites for SH3 domains, a module present in a number of proteins believed to mediate protein–protein interactions that are important in signal transduction (Olivier *et al.*, 1993; Ren *et al.*, 1993). The proline-rich areas in KV3.2 and KV3.3 contain stretches that fit the proposed consensus sequences for SH3-domain binding. Our laboratory is currently investigating the interaction of the proline-rich domains of KV3.2 and KV3.3 with SH3-domain-containing proteins.

6. Subunit Association Domains

The amino acid sequence of the N terminal region of *Sh*III proteins that follows the N-terminal inserts in KV3.3 and KV3.4 is extremely well conserved (see Fig. 11). The sequences are also very similar to the equivalent sequence in the *Drosophila Shaw* protein (Vega-Saenz de Miera *et al.*, 1992; see also Table VI). In analogous positions of other *Sh* proteins, the amino acid sequence is very well conserved within members of the same subfamily, but shows relatively little conservation among members of different subfamilies (Stühmer *et al.*, 1989a; Jan and Jan, 1990a; Perney and Kaczmareck, 1990; Baldwin *et al.*, 1991; Rudy *et al.*, 1991a; Hwang *et al.*, 1992; Pongs, 1992). In *Sh*III proteins, the region of similarity is broken into two conserved areas by the polyproline insert of KV3.2 (see Fig. 11).

Li *et al.* (1992) and Shen and Pfaffinger (1992) showed that the conserved area prepared *in vitro* can form tetramers or bind to *in vitro* translated subunits of the same subfamily but not of different subfamilies. These and other elegant experiments provide evidence that the self-aggregating conserved area includes domains (hereafter called subunit recognition domains) that are important for both subunit recognition and channel formation, and may be the basis for the formation of heteromultimeric channels by subunits of the same subfamily. Additional evidence that these subunit recognition domains are crucial for the assembly of appropriate subunits was provided by Li *et al.* (1992), who used chimeric proteins to show that these domains were sufficient to determine co-assembly of subunits into functional channels.

7. *N*-Glycosylation Sites in the Linker between the S1 and S2 Domains

All *Sh*III proteins have two putative *N*-glycosylation sites in the linker between the S1 and the S2 domains that are thought to face the extracellular surface of the membrane (indicated with triangles in Fig. 11). In

KV3.3, another putative *N*-glycosylation site is found in the linker between the S5 and H5 domains (see Fig. 11). Whether these sites are indeed glycosylated in *Sh*III proteins, and the functional consequences, if any, of this glycosylation, are not yet known. Most *Sh*I proteins also contain a putative *N*-glycosylation site in the S1–S2 linker, whereas *Sh*II proteins have an *N*-glycosylation site in the S3–S4 linker.

8. Disruption of the Leucine-Heptad Repeat in *Sh*III Proteins

When the sequence of the S4 domain, the linker between the S4 and S5 domains, and the beginning of the S5 domain of *Sh* proteins is drawn in α-helical form, one side of the α helix is particularly well conserved (Fig. 14). In particular, conservation of one arm of the helix (number 1 in Fig. 14), consisting of the third arginine in S4 and five leucine residues, spaced 7 amino acids apart, is apparent (K. McCormack *et al.*, 1989,1991). Mutagenesis of these residues leads to profound changes in voltage-dependent gating (K. McCormack *et al.*, 1991; Schoppa *et al.*, 1992). K. McCormack *et al.* (1991) proposed that the leucines in the heptad repeat mediate hydrophobic interactions that are crucial to the conformational changes involved in voltage-dependent gating.

Interestingly, in all *Sh*III proteins the fourth leucine of the leucine heptad repeat is substituted by phenylalanine. Researchers have suggested that this substitution might explain why *Sh*III channels require very positive potentials for activation (T. McCormack *et al.*, 1990; Rudy *et al.*, 1991a,b; Vega-Saenz de Miera *et al.*, 1992). This hypothesis has only been tested indirectly. Rettig *et al.* (1992) reported that replacing the phenylalanine with leucine and the next residue in the sequence (a leucine in all *Sh*III proteins) with glycine (making this area similar to *Shaker*-related proteins) produced a 16-mV negative shift in the voltage-dependence of a KV3.4 channel. The interpretation of their results is not straightforward since two residues were changed. Further, the interpretation is complicated by the fact that Rettig *et al.* (1992) utilized a KV3.4 cDNA with a spontaneous mutation in the S4 domain (H364Q), which itself produced a large positive shift in the voltage dependence of this channel relative to normal KV3.4 channels. Nevertheless, this result is encouraging and stimulates interest in further mutagenesis studies.

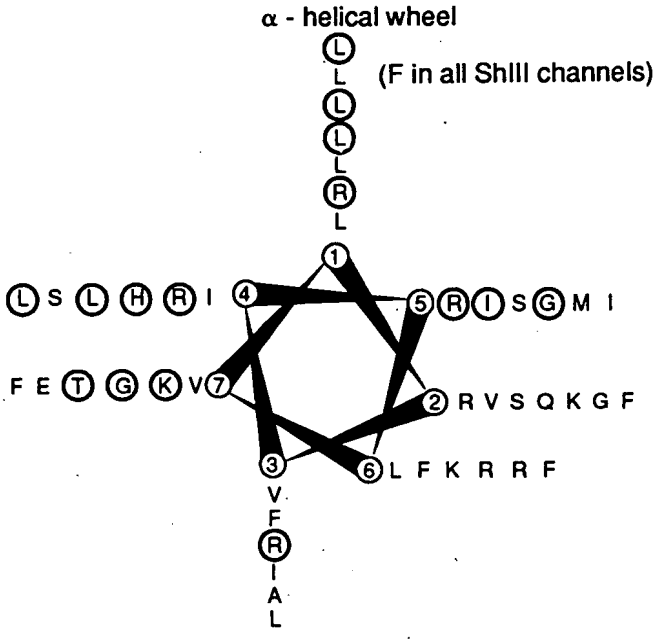


FIGURE 14 Helical representation of the S4–S5 region of the KV1.1 protein. The sequence of the KV1.1 protein, starting at the second residue in S4 (a leucine) and ending in the last leucine of the leucine heptad repeat (located in the S5 domain), has been projected onto an α-helical wheel. Residues that are conserved among all mammalian *Sh* proteins have been circled.

9. Cluster of Positively Charged Residues After the S6 Domain

All *Sh*III proteins possess a cluster of positively charged residues just after the S6 domain region (indicated with a star in Fig. 11). A similar sequence is found (see Fig. 12D) in the two inward rectifier K^+ channels IRK1 (Kubo *et al.*, 1993) and ROMK1 (Ho *et al.*, 1993). These similarities are intriguing. Perhaps these structural similarities are related to the rectification seen in *Sh*III channels (see Section IV,D).

10. Carboxyl Ends

*Sh*III proteins become particularly variable after the common cluster of positive charges found after the S6 domain. Moreover, all *Sh*III proteins terminate in divergent sequences derived by alternative splicing. Nevertheless, some short stretches of highly conserved amino acid sequence are seen, particularly in the portions of the protein thought to be encoded by exon 3. As an example, consider the sequence PLAQEEI (residues 489–495) in KV3.1, PLAQEEV (residues 648–654) in KV3.3, and PPAREEG in KV3.4 (residues 526–532) (see Fig. 11).

11. Putative Phosphorylation Sites

Several sites that fit the consensus sequence for protein kinase phosphorylation are found in regions of *Sh*III proteins that are thought to be intracellular. Some of these sites are found in the products of all the genes, some are found only in proteins encoded by a single gene, and some are specific to putative alternatively spliced isoforms. The consensus sites for three kinases are listed in Table IV. None of these

TABLE IV **Putative Sites for Protein Kinase Phosphorylation in *Sh*III Channels**[a]

			KV3.1	KV3.2	KV3.3	KV3.4
Putative sites for protein kinase C	Sites common to all *Sh*III channels	Threonine	21, 25, 73, 108, 337	21, 25, 155, 374	103, 107, 155, 190, 441	49, 53, 113, 148, 374
	Gene-specific sites	Serine	185	224	286	222
		Threonine	152		16, 657	
		Serine		205, 564	11, 89	9, 35, 515
	Sites specific to an alternatively spliced version			KV3.2d threonine 609	KV3.3a serine 731; 769	KV3.4a, KV3.4c serine 604
Putative sites for protein kinase A	Sites common to all *Sh*III channels	Threonine	21, 25, 342	21, 25, 379	103, 107, 446	49, 53, 379
		Serine	341	378	445	378
	Gene-specific sites	Threonine		574	16	520
		Serine		563, *564*		15, 519
	Sites specific to an alternatively spliced version		KV3.1a serine 510, KV3.1b serine 503, threonine 539, 585	KV3.2a threonine 613, KV3.2d serine 613, KV3.2b serine 615	KV3.3a threonine *736*, 852, 865, serine 713, 885, 886	KV3.4a, KV3.4c serine 541, 578, KV3.4c threonine *609*
Putative sites for casein kinase II	Sites common to all *Sh*III channels	Threonine	99	146	181	139
		Serine	341	378	445	378
	Gene-specific sites	Threonine	483	17, 520, 546, 579	120	45, 520
		Serine	44, 130	414, 541, 553, 564		515
	Sites specific to an alternatively spliced version		KV3.1b threonine 527, serine 550	KV3.2a threonine 605	KV3.3a serine 697	KV3.4a, KV3.4c threonine 568, serines 552, 594, KV3.4c threonine 609

[a] The deduced amino acid sequences of *Sh*III channels contain several intracellular sites that fit the consensus sequences of protein kinase A (R-[R/K]-X-[T/S] >> R-X_2-[S/T] = R-X-[S/T]; Kemp and Pearson, 1990; Kennelly adn Krebs, 1991; some of these sites may be phosphorylated as well by cGMP-dependent protein kinase); protein kinase C ([R/K_{1-3}]-X_{2-0}-[S/T]-X-[R/K] or [S/T]-X-[R/K]; Kemp and Pearson, 1990; Kennelly and Krebs, 1991; sites where the S or T is separated by more than one residue from the C-terminal basic residue have not been included); and casein kinase II ([S/T]-X_2-[D/E]; (Marin *et al.*, 1986; additional putative sites for this kinase are not included; see Kennelly and Krebs, 1991). The numbers represent the positions of the amino acids that are putatively phosphorylated. We have grouped these sites into three different categories: (1) sites common to all *Sh*III channels, that is, sites that are in a similar position when all *Sh*III polypeptides are aligned to maximize aa identity; (2) gene-specific sites, that is, sites only found in the proteins encoded by a given *Sh*III gene; and (3) sites specific to alternatively spliced versions, which are found in the region of divergence of the alternatively spliced products. For protein kinase A, the numbers in italics correspond to sites with a better consensus sequence (R-[R/K]-X-[T/S]; Kennelly and Krebs, 1991). The influences of residues near the putative recognition sequence motif (Kemp and Pearson, 1990) were not considered.

sites has been shown to be phosphorylated, although Critz *et al.* (1993) reported inhibition of a KV3.1 channel by phorbol esters that activate protein kinase C and we have seen inhibition of KV3.2 currents by cAMP-dependent phosphorylation (H. Moreno and B. Rudy, unpublished observations).

Since alternatively spliced variants express nearly identical currents in *Xenopus* oocytes (see Section IV,D), and since they also appear to have similar expression patterns in brain (see Section IV,H), the existence of transcript-specific putative phosphorylation sites is especially interesting.

H. Tissue and Cellular Distribution of ShIII Transcripts

Studies of the distribution of *Sh*III mRNAs utilizing Northern blot and RNAse protection analysis, as well as *in situ* hybridization histochemistry (Drewe *et al.*, 1992; Perney *et al.*, 1992;[3] Rudy *et al.*, 1992; Weiser *et al.*, 1994), have revealed a number of interesting features of the tissue distribution of *Sh*III transcripts in rat. In addition, these studies have provided important clues about which subunit combinations might occur *in vivo*. Although the specific localization of *Sh*III proteins remains to be determined, the mRNA localizations suggest where to begin searching for native channels containing *Sh*III proteins using electrophysiological methods.

A Northern blot of mRNA isolated from various rat tissues hybridized with *Sh*III gene-specific probes is shown in Fig. 6. The probes used in this experiment differentiate among the products of different genes but not among alternatively spliced variants of the same gene. KV3.1, KV3.2, and KV3.3 are expressed mainly in the brain. Low levels of KV3.1 transcripts (mainly the 8-kb band) are also seen in skeletal muscle, whereas low levels of KV3.3 mRNAs are found in kidney and lung. According to Ghanshani *et al.* (1992), KV3.3 transcripts are also present in mouse liver, thymus, and heart. In contrast, KV3.4 transcripts are more abundant in skeletal muscle than in brain. KV3.1 (KV3.1a only), KV3.3, and KV3.4 products have also been identified in PC12 pheochromocytoma cells (Vega-Saenz de Miera *et al.*, 1991,1992; Ried *et al.*, 1993). KV3.1 transcripts were also found in T lymphocytes, in which they are thought to be a primary component of the L-type K^+ channel and in spleen (Grissmer *et al.*, 1992).

[3] KV3.1α and KV3.1β in Perney *et al.* (1992) correspond to KV3.1b and KV3.1a, respectively, in Weiser *et al.* (1994) and in this chapter.

Quantitative analysis (Weiser *et al.*, 1994) demonstrated that, in the adult rat brain, KV3.1 (KV3.1b) and KV3.3 transcripts were the most abundant *Sh*III mRNAs. They appeared to be present in amounts similar to KV1.2 mRNAs, a *Shaker*-related transcript (Chandy *et al.*, 1991). KV3.1 (KV3.1b) and KV3.3 mRNAs were ~3 times more abundant than KV3.2 mRNAs, ~6 times more abundant than KV3.4 transcripts, and about 1/6 as abundant as Na^+ channel α subunit mRNAs.

1. *Sh*III Gene Expression during Development

Little is known about the expression of *Sh*III genes during development. Perney *et al.* (1992) found that low levels of KV3.1 transcripts were detected in the rat brain as early as embryonic day 19. By postnatal day 3 expression was detected in the cortex and in the hippocampus. A large increase in expression of these transcripts was seen, however, between the 7th and 14th day after birth. In fact, postnatal day 14 rats exhibited a pattern of expression in brain similar to that seen in the adult rat. KV3.2 mRNAs were also detectable in postnatal day 1 rat brains, but increase several-fold between day 10 and 15 (C. Kentros, unpublished observations).

2. Specific but Overlapping Expression of *Sh*III mRNAs in the CNS

In situ hybridization histochemistry has been used to study the distribution of *Sh*III mRNAs in the central nervous system (CNS) of adult rats (Drewe *et al.*, 1992; Perney *et al.*, 1992; Rettig *et al.*, 1992; Rudy *et al.*, 1992; Weiser *et al.*, 1994). The work by Perney *et al.* (1992) on KV3.1 and the work by Weiser *et al.* (1994) on KV3.1, KV3.2, KV3.3, and KV3.4 include, in addition to low-resolution X-ray autoradiography (which provides regional localization), a higher cellular-resolution microscopic analysis of emulsion-dipped sections. The data from these two studies form the basis for the descriptive analysis presented here.

As is the case with neurotransmitters, receptors, and other molecular markers, *Sh*III mRNAs were distributed in a nonhomogeneous fashion throughout the CNS. In fact, some neuronal types (e.g., most neurons in the cerebral cortex, the caudate–putamen, the amygdala, the epithalamus, the hypothalamus, the substantia nigra compacta, and a few structures in the brain stem such as the inferior olive) did not appear to express significant amounts of any *Sh*III mRNAs. Interestingly, the main neuronal population of the caudate–putamen appears to express few *Sh* transcripts of the other subfamilies as well (Weiser *et al.*, 1994). Of those tested, only probes for KV1.4 of

the *Sh*I subfamily (Sheng *et al.*, 1992) and KV4.2 of the *Sh*IV subfamily (Tsaur *et al.*, 1992) labeled the caudate–putamen strongly. Only weak labeling was seen with *Sh*II probes (Drewe *et al.*, 1992; Hwang *et al.*, 1992).

Based on the distribution of hybridization signals as well as on cell size and morphology of labeled cells in Nissl-counterstained sections, Perney *et al.* (1992), Rudy *et al.* (1992), and Weiser *et al.* (1994) concluded that in the CNS *Sh*III mRNAs were present mainly in neurons and not glia. In all cases, hybridization signals were seen in the larger diameter cells with characteristics of neuronal somata. Moreover, highly sensitive RNAse protection assays with RNA from glial cells cultured from rat neonate optic nerve and from C6 glioma cells did not detect KV3.1 transcripts (Perney *et al.*, 1992).

In the rat CNS, each *Sh*III gene was found to exhibit a unique pattern of expression. However, a significant degree of overlap was seen in the distribution of KV3.1 and KV3.3 mRNAs. Moreover, KV3.4 transcripts were only present, albeit at lower levels, in several of the neuronal populations that also expressed KV3.1 and/or KV3.3 mRNAs. The expression patterns of transcripts of the four *Sh*III genes in adult rat brain are illustrated diagramatically in Fig. 15.

KV3.1, KV3.3, and KV3.4 exhibited patterns of expression more similar to each other than that of KV3.2 transcripts, thus defining two trends in the expression of *Sh*III genes in the CNS. Many neurons expressing KV3.2 mRNAs expressed these transcripts predominantly (relative to the other *Sh*III transcripts), if not exclusively (e.g., in thalamic relay neurons of many nuclei of the dorsal thalamus, and in neurons of the optic layer of the superior colliculus and the locus coeruleus). At the same time, many regions that expressed KV3.1, KV3.3, or KV3.4 mRNAs prominently—such as the cerebellar cortex, the spinal cord, the reticular thalamic nucleus, the inferior colliculus, and many nuclei in the brain stem—appeared to express little or no KV3.2 transcripts.

Other interesting features of *Sh*III mRNA expression were the differences between dorsal and ventral thalamus (Fig. 15). KV3.2 mRNAs were most abundant in thalamic relay neurons throughout the dorsal thalamus, but were weakly expressed in the reticular thalamic nucleus, a structure of the ventral thalamus. On the other hand, KV3.1 and KV3.3 mRNAs were much more abundant in the neurons of the reticular thalamic nucleus than in thalamic relay neurons of the dorsal thalamus. These two parts of the thalamus have been demonstrated to have different embryological origins (Rose, 1942) and are functionally distinct, since only the nuclei in the dorsal thalamus project to the cerebral cortex (Jones, 1985; Steriade *et al.*, 1990).

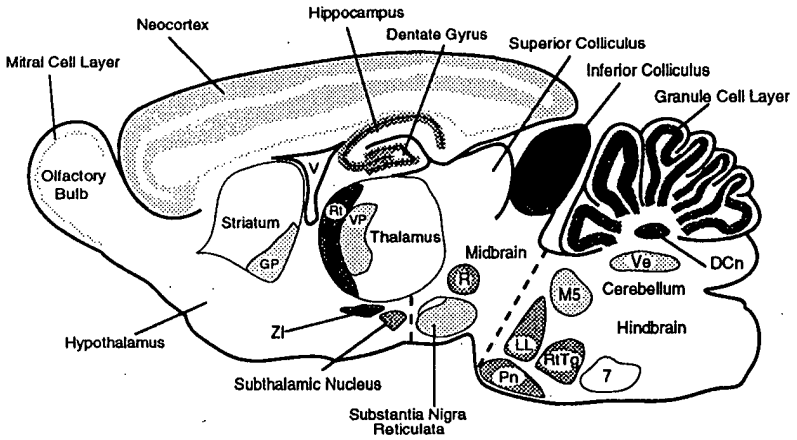
3. Transcript-Specific Expression

The study by Weiser *et al.* (1994) used gene-specific probes that did not distinguish among the alternatively spliced variants of each gene. In two cases, KV3.1 (Perney *et al.*, 1992) and KV3.2 (Rudy *et al.*, 1992), *in situ* hybridization with probes specific to alternatively spliced variants produced spatially identical labeling patterns, suggesting that the variants of each gene are colocalized to the same neurons. Perney *et al.* (1992), however, noted differences in the developmental expression of KV3.1a and KV3.1b transcripts in the rat brain. KV3.1a transcripts were more abundant prenatally and at earlier postnatal periods, whereas the reverse was true in the adult brain.

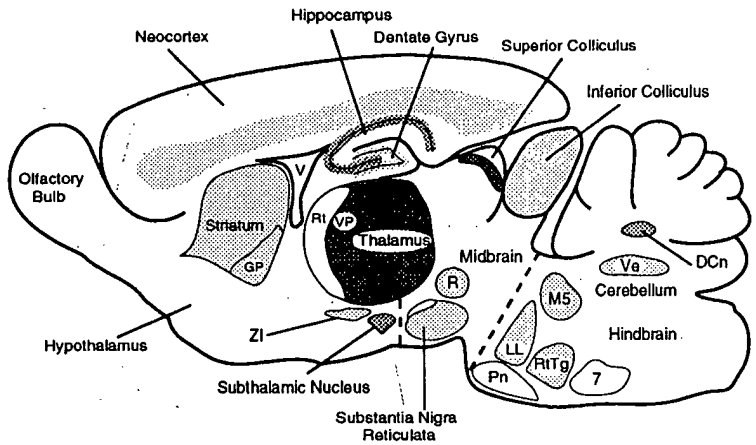
4. *Sh*III mRNA Expression Distinguishes among Subsets of Interneurons in the Cortex, Hippocampus, and Caudate Putamen

*Sh*III probes produced patchy labeling in the cerebral cortex and the hippocampus (Perney *et al.*, 1992; Weiser *et al.*, 1994). Higher magnification studies in these areas showed that these signals were caused by hybridization in a small number of neurons that were morphologically indistinguishable from other cells in the area. In addition, *Sh*III probes labeled a unique subset of neurons in the caudate–putamen (Weiser *et al.*, 1994). Therefore, subsets of neurons in these regions apparently can be distinguished by their expression of *Sh*III mRNAs.

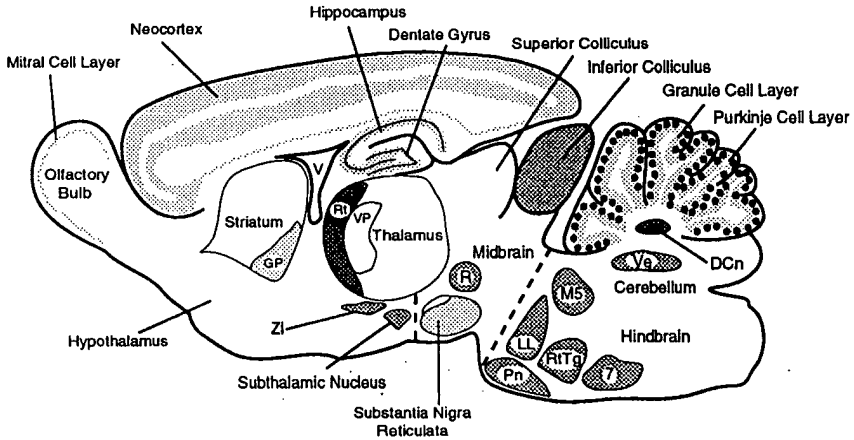
Based on distribution and cell morphology, Perney *et al.* (1992) and Weiser *et al.* (1993) concluded that the cells expressing *Sh*III mRNAs in the neocortex were local circuit neurons which, in the rat cortex, represent a small percentage of the total neuronal population. KV3.2 mRNAs were present in neurons located in layers IV–VI, resembling the distribution of D1 dopamine receptors and somatostatin binding (Weiser *et al.*, 1994). KV3.1 mRNAs and, to a lesser degree, KV3.3 and KV3.4 mRNAs were present in cells located throughout layers II–VI, being more numerous in layer II and in the superficial parts of layers III and IV. Thus, in X-ray autoradiograms, probes specific for KV3.2 mRNAs produced a band localized toward deep layers of the cortex, whereas KV3.1, KV3.3, and KV3.4 probes produced a double band: one in superficial layers, which was more intense, and one in deeper layers (Perney *et al.*, 1992; Weiser *et al.*, 1994).



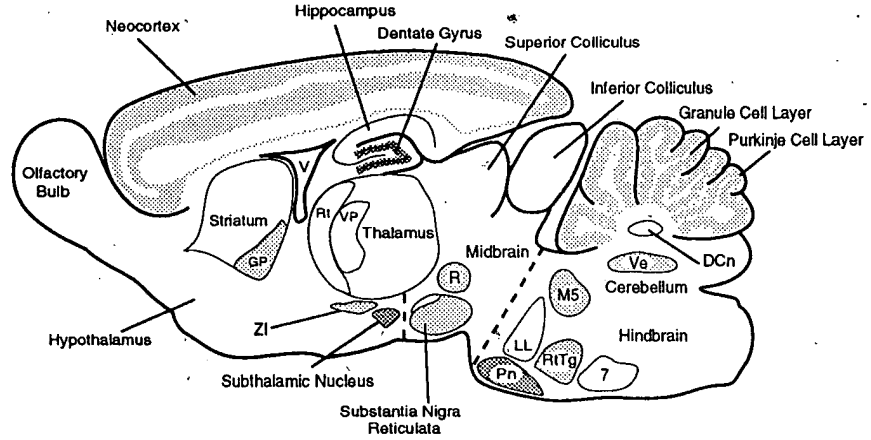
KV3.1



KV3.2



KV3.3



KV3.4

*Sh*III mRNAs were weakly to moderately expressed in the pyramidal and granule cells of the hippocampus (Fig. 15). In addition, KV3.1, KV3.2, and KV3.3 mRNAs were also expressed in specific subsets of interneurons (Perney *et al.*, 1992; Weiser *et al.*, 1994). The differences in labeled interneuron distribution were more notable in the CA1 and hilar regions. The distribution of cells expressing KV3.2 mRNAs was similar to that reported for somatostatin immunoreactivity (Weiser *et al.*, 1994). Labeled cells were numerous in the stratum oriens in the CA1 region as well as in the hilus. In contrast, the distribution of cells hybridizing with KV3.1 and KV3.3 probes resembled that for GABA or cholecystokinin (Perney *et al.*, 1992; Weiser *et al.*, 1994). In the CA1 region, labeled cells were seen along the stratum radiatum–pyramidalis border and along the stratum oriens–pyramidalis border. In the area dentata, KV3.1- and KV3.3-labeled cells were more numerous in the borders of the granular cell layer than in the hilus proper. Subsets of cortical and hippocampal interneurons have been described based on differences in morphology (DeFelipe and Jones, 1988; Zilles, 1990) or in the expression of various neurotransmitters, neuropeptides, and receptors (Somogyi *et al.*, 1984; Douglas and Martin, 1990; Lin *et al.*, 1986; Sloviter and Nilaver, 1987; Demeulemeester *et al.*, 1988; Woodson *et al.*, 1989; Zilles *et al.*, 1990).

In the caudate–putamen, *Sh*III probes labeled a very small number of neurons (Weiser *et al.*, 1994) that were described to be a subset of small to medium-sized cells distinct from the large cholinergic local circuit neurons. Weiser *et al.* (1994) hypothesized that the *Sh*III mRNA-containing neurons were a subpopulation of the principal GABAergic neurons that comprise over 90% of the cells in the caudate–putamen, or perhaps yet another distinct subpopulation of interneurons.

5. Coexpression of *Sh*III Transcripts in the Same Neuronal Populations Reveals a High Potential for Heteromultimer Formation

Based on compelling evidence from overlapping labeling with multiple probes in easily identifiable neuronal populations, Weiser *et al.* (1994) concluded that neurons in several CNS areas are likely to coexpress transcripts of more than one *Sh*III gene. These investigators argued that heteromultimer formation is a potentially significant feature of the channels formed by subunits of the *Shaw* subfamily and raised the interesting possibility that the subunit composition of heteromultimers could vary in different neurons since the ratio of overlapping signals changed from one neuronal population to another.

The neuronal populations that may coexpress transcripts of distinct *Sh*III genes include the mitral cells of the olfactory bulb; the principal neurons of the reticular thalamic nucleus, the lateral leminiscus, the reticulo-tegmental nucleus of the pons, and the trigeminal motor nucleus, which were labeled with

FIGURE 15 Distribution of *Sh*III mRNAs in the rat brain. The levels of expression of transcripts from four different *Sh*III genes, based on *in situ* hybridization histochemistry studies by Perney *et al.* (1992) and Weiser *et al.* (1994), are represented by different grades of shading. KV3.1 and KV3.3 mRNAs were most prominent in the cerebellar cortex, but are also abundant in the reticular thalamic nucleus (Rt), the inferior colliculus, and several nuclei in the brain stem, including the nuclei of the lateral lemniscus (LL), the reticulo-tegmental nucleus of the pons (RtTg), the pontine nuclei (Pn), the trigeminal motor nuclei (M5), and the vestibular nuclei (Ve). These RNAs were also expressed prominently in the cochlear nucleus and spinal cord (not shown). Moderate to low expression was seen in the mitral cells of the olfactory bulb and in thalamic relay neurons of some nuclei of the dorsal thalamus (such as the ventral posterior complex, VP). Hybridization signals with KV3.2 probes were strongest in thalamic relay neurons throughout the dorsal thalamus. KV3.2 signals were also prominent on neurons of the optic layer of the superior colliculus. Moderate to weak hybridization was seen in the locus coeruleus and a few other structures in the brain stem. KV3.4 mRNAs were the least abundant *Sh*III RNA in the central nervous system. They were seen mainly in areas that also contain KV3.1 and/or KV3.3 transcripts, usually at higher levels.

All four *Sh*III mRNAs are expressed in the giant neurons of the gigantocellular part of the red nucleus (R) and in the majority of the neurons of the subthalamic nucleus. All were present as well in parts of the septum, zona incerta (ZI), substantia nigra reticulata, globus pallidus (GP), reticulo-tegmental nucleus of the pons (RtTg), and oculomotor nucleus. KV3.1, KV3.3 mRNAs, and, to a lesser degree, KV3.2 mRNAs were expressed in the large neurons of deep cerebellar nuclei (DCn). Each *Sh*III mRNA had a characteristic distribution in the hippocampus. KV3.1, KV3.2, and KV3.3 mRNAs, but not KV3.4 mRNAs, were expressed weakly in the pyramidal cell layers of the CA1–CA3 fields. KV3.1, KV3.4, and, to a lesser degree, KV3.3 mRNAs were expressed in granule cells of the dentate gyrus. KV3.1, KV3.2, and KV3.3 mRNAs also were expressed in distinct subsets of hippocampal interneurons. See text and original references for further details.

KV3.1 and KV3.3 probes; and neurons of the pontine nuclei and some cerebellar Purkinje cells, which were labeled with KV3.1, KV3.3, and KV3.4 probes. Coexpression of KV3.1 and KV3.3 is also likely in the inferior colliculus and the reticular nuclei of the hindbrain, although the complex cytoarchitecture of these areas made it more difficult for Weiser *et al.* (1994) to assess whether cells were co-labeled.

Other neuronal populations appeared to express all *Sh*III mRNAs, albeit at different levels. These groups included the principal neurons of the globus pallidus, the subthalamic nucleus, the diagonal band of Broca, the substantia nigra pars reticulata, the zona incerta, and the giant neurons of the gigantocellular part of the red nucleus (see Fig. 15). In addition, thalamic relay neurons of some nuclei of the dorsal thalamus were found to coexpress KV3.2, KV3.1, and KV3.3 mRNAs.

6. Correlation between Expression Patterns of *Sh*III mRNAs and Other Neuronal Markers

Many, but by no means all, neurons expressing *Sh*III transcripts, particularly KV3.1, KV3.3, and KV3.4 mRNAs (e.g., interneurons in the cerebral cortex and the hippocampus; the neurons of the reticular thalamic nucleus, globus pallidus, zona incerta, inferior colliculus, nuclei of the lateral lemniscus, and the substantia nigra pars reticulata; and cerebellar Purkinje cells) are GABAergic, a feature first noted by Perney *et al.* (1992) for KV3.1 mRNAs. Although this extensive correlation is intriguing, note that some important GABAergic systems, notably the main neuronal population of the caudate–putamen and the olfactory bulb, do not appear to express these mRNAs. Moreover, some cells that express the mRNAs prominently, such as granule cells in the cerebellum and thalamic relay neurons, are not GABAergic but do receive GABAergic input. Perhaps channels containing these subunits mediate the effects of putative $GABA_B$ receptors.

The resemblance between the distribution of KV3.2 mRNAs in the cerebral cortex and the localization of somatostatin receptors is also interesting. Somatostatin has been shown to modulate a voltage-dependent K^+ current in cultured rat neocortical neurons that resembles KV3.2 currents in *Xenopus* oocytes in voltage dependence and kinetics (Wang *et al.*, 1989).

Other than these similarities, no obvious common characteristics of the neurons expressing any or all *Sh*III transcripts were found. Such a correlation would have provided strong hints about the functional role of *Sh*III subunits. However, the lack of correlation is not necessarily surprising. There are multiple receptors for most neurotransmitters and neuropeptides, each acting on different second messenger cascades. In addition to this divergence, there is a convergence of different neurotransmitter and neuropeptide receptors eliciting the same second messenger response. Further, a given voltage-gated K^+ channel could play different roles in different neurons or even in different places in the same neuron. In some cells, this channel may mediate the response to one neurotransmitter, whereas in another cell it could mediate the response to another neurotransmitter acting on the same second messenger system. Moreover, the same channel could be affected by more than one kinase and, hence, mediate the effect of a different second messenger system in a third group of cells, and play a role as a voltage-gated channel independent of second messenger modulation in another group of cells.

I. Searching for Candidate Native ShIII *K^+ Channels*

One approach to identifying native channels containing cloned K^+ channel subunits is to search for *in vivo* currents that resemble the currents expressed by these subunits in heterologous expression systems. Using such a strategy, investigators have found some attractive candidates, although adequate methods to prove unequivocally that the candidate channels contain a given subunit are unavailable. For example, Pardo *et al.* (1992) found that an A current recorded in rat cultured hippocampal neurons resembled the currents expressed *in vitro* by KV1.4 transcripts, of the *Shaker*-related subfamily, in voltage dependence, pharmacology, and kinetics, including a slow recovery from inactivation. Moreover, the native current was greatly reduced when the cell was perfused with K^+-free solution, returning to normal when the external K^+ was restored. Among several *Sh* channels tested in *Xenopus* oocytes, only KV1.4 channels were similarly affected by K^+-free solutions (Pardo *et al.*, 1992).

Another example is the N-type K^+ channel in T lymphocytes, which resemble the channels expressed in *Xenopus* oocytes by KV1.3 transcripts, also of the *Shaker*-related subfamily (Grissmer *et al.*, 1990), in many macroscopic and microscopic properties. Moreover, KV1.3 channels in *Xenopus* oocytes were the only known cloned K^+ channel blocked by margatoxin, a peptide derived from the venom of the scorpion *Centruroides margaritus*, which also blocks the N-type K^+ channel in lymphocytes (Novick *et al.*, 1991; Leonard *et al.*, 1992).

A different and interesting situation is encountered in the case of channels of the *Shal*-related subfamily.

A *Shal*-related transcript, KV4.2, expresses currents in *Xenopus* oocytes that resemble the low voltage-activating A currents that have been recorded in several neuronal types (Baldwin *et al.*, 1991; Pak *et al.*, 1991) and those seen in *Xenopus* oocytes injected with rat brain poly(A) mRNA (Rudy *et al.*, 1988). KV4.2 currents activate and inactivate near neuronal resting potentials, are insensitive to TEA but are blocked by millimolar concentrations of 4-AP, and have macroscopic time constants of inactivation that do not change with the membrane potential. However, KV4.2 currents in *Xenopus* oocytes recover very slowly from inactivation unlike typical low voltage-activating A currents found in neurons or those seen in oocytes injected with rat brain mRNA. Antisense hybrid-arrest experiments demonstrate that KV4.2 subunits indeed constitute a main component of the low voltage-activating A current that is expressed in *Xenopus* oocytes injected with rat brain mRNA (Serodio *et al.*, 1992). Serodio *et al.* (1992) suggested that additional proteins encoded in total rat brain mRNA (perhaps other channel subunits or modifying enzymes) are responsible for the differences between KV4.2 channels and those expressed from brain mRNA. Native channels might be similarly modified by these putative additional proteins. Chabala *et al.* (1992) also obtained evidence for the existence of modifying factors for another *Shal*-related transcript, KV4.1, that is much less abundant in brain than KV4.2 (P. Serodio and B. Rudy, unpublished observations). The situation encountered with these *Sh* channels is reminiscent of that of the rat brain Na^+ channel α subunit which, when expressed in *Xenopus* oocytes, has inactivation properties that are different from those of native Na^+ channels. Co-injection with rat brain Na^+ channel β subunits results in the expression of channels that more closely resemble native Na^+ channels (Isom *et al.*, 1992).

Regarding the identification of native K^+ channels containing *Sh*III subunits, Grissmer *et al.* (1992) proposed that the L-type K^+ channel in T lymphocytes is probably a homomultimer of KV3.1 subunits. These workers found that macroscopic KV3.1 currents in oocytes were indistinguishable from those of the L-type channel in T cells expressing KV3.1 RNAs. To eliminate differences resulting from different solutes across the membrane, Grissmer *et al.* (1992) made all their recordings in excised patches using identical solutions across the patch. The single-channel conductance of KV3.1 channels in *Xenopus* oocytes under the recording conditions used (27 pS) was also found to be very similar to that of L-type K^+ channels in mouse and human T lymphocytes (21–27 pS). Moreover, KV3.1 RNA was not found in cells containing little or no L-type channels, such as the EL4 mouse T cell line (Grissmer *et al.*, 1992).

Another native K^+ channel proposed to contain *Sh*III subunits (Vega-Saenz de Miera and Rudy, 1992) is the O_2-sensitive K^+ channel present in type I cells of the carotid body (Lopez-Barneo *et al.*, 1988; Lopez-Lopez *et al.*, 1989; Urena *et al.*, 1989; Ganfornina and Lopez-Barneo, 1991,1992a,b). Lopez-Barneo and colleagues hypothesized that the modulation of the activity of this K^+ channel by changes in arterial O_2 tension is one of the initial triggering events of the sensory response to changes in pO_2 in the carotid body (Lopez-Barneo *et al.*, 1988; Lopez-Lopez *et al.*, 1989; Urena *et al.*, 1989; Ganfornina and Lopez-Barneo, 1991, 1992a,b). The Po_2-sensitive current in type I cells of the carotid body is a TEA-sensitive, voltage-dependent, transient K^+ current that activates when the membrane potential is more positive than −20 mV. Of all cloned K^+ channels, only two *Sh*III channels—KV3.3 and KV3.4—express transient, TEA-sensitive, voltage-dependent K^+ currents requiring voltages more positive than −20 mV to activate in *Xenopus* oocytes. The kinetics of the pO_2-sensitive current, especially its time course of inactivation, are particularly similar to the currents expressed by KV3.3 mRNAs (KV3.4 currents inactivate much more rapidly). However, heteromultimers of KV3.4 and other *Sh*III proteins have intermediate inactivation properties (see Section IV,E); therefore, heteromultimers of KV3.4 and KV3.3 or KV3.4 with *Sh*III proteins expressing noninactivating channels could also produce currents similar to those of the pO_2-sensitive channel. Interestingly, PCR experiments demonstrate that KV3.3 mRNAs are present in the rabbit carotid body (E. Vega-Saenz de Miera and B. Rudy, unpublished observations). The pO_2-sensitive channel has been well characterized at the single-channel level (Ganfornina and Lopez-Barneo, 1991,1992a,b). Microscopic analysis of KV3.3 channels (or other *Sh*III channels) in heterologous expression systems may allow a more detailed comparison with the native channel.

Ito *et al.* (1992) recorded oocytes injected with KV3.1a cRNA with long (5 sec) depolarizing pulses to characterize the slow inactivation of KV3.1a currents and to compare these currents with a slowly inactivating current found in NG108-15 cells (Robbins and Sims, 1990) expressing KV3.1a mRNAs (Yokoyama *et al.*, 1989). Although the NG108-15 currents showed some resemblance to KV3.1a currents in oocytes, they began activating at more negative voltages and are much less sensitive to external TEA.

Given the results of mRNA expression studies, one would expect to find *Sh*III channels in several CNS neurons. However, the most notable feature is the

general lack of resemblance of *in vitro* *Sh*III channels to typical K^+ currents seen in somatic recordings of neurons. For example, KV3.2 mRNAs appear to be most abundant in thalamic relay neurons. Two delayed rectifier currents, I_{K1} and I_{K2}, and a low voltage-activating transient current have been described in acutely dissociated thalamic relay neurons (Hugenard and Prince, 1991). None of these currents shows properties identical to those of KV3.2 channels in *Xenopus* oocytes (or those expected of any KV3.2 heteromultimeric combination). I_{K1}, which accounts for only 2% of the total outward current, is similar to KV3.2 channels in voltage dependence and sensitivity to TEA, but is proposed to be Ca^{2+} activated and is more sensitive to 4-AP, whereas I_{K2} activates at more negative voltages and is less sensitive to TEA.

Attempts at similar comparisons between the K^+ currents recorded in cerebellar Purkinje cells (Bossu *et al.*, 1988; Gähwiler and Llano, 1989; Hirano and Ohmori, 1986) and the currents expressed in oocytes by the *Sh*III transcripts present in these cells (KV3.3 >> KV3.4 > KV3.1) present difficulties that may explain why we are unable to find many clear-cut examples of *in vivo* currents in neurons expressing *Sh*III mRNAs similar to those of *in vitro* *Sh*III channels. The isolation of individual components of the total K^+ current is often tailored to the goals of a particular investigation. If the native *Sh*III currents in a given cell do not constitute a major portion of the total K^+ current, they could be masked by currents with overlapping electrophysiological and pharmacological properties. For example, none of the currents described in cultured cerebellar Purkinje cells is very similar to the currents expressed in oocytes by KV3.3 RNAs (which are abundant in cerebellar Purkinje cells) or by mixtures of KV3.3 and KV3.4 or KV3.1 RNAs. However, KV3.3 currents could account for some of the inactivating component of the "maintained," TEA-sensitive outward current seen in Purkinje cells in organotypic cultures, or some of the slow components of the transient current (Gähwiler and Llano, 1989). Because of the difficulties in isolating K^+ current components, electrophysiological experiments specifically designed to search for native channels with properties similar to those of *Sh*III channels *in vitro* might be required before considering other reasons for the apparent lack of correspondence between native and oocyte currents.

At least three additional explanations are possible for the apparent lack of oocyte-like *Sh*III currents in neurons expressing *Sh*III mRNAs. First, voltage-clamp studies from neurons often use dissociated embryonic or early postnatal tissue, which may not express *Sh*III subunits. Second, native channels containing *Sh*III proteins might have, at least in some cell populations, electrophysiological properties different from those of *in vitro* *Sh*III channels because of as yet unknown factors (additional subunits, posttranslational modifications, membrane lipid composition, etc.). Finally, *Sh*III channels might be more prominent in neuronal processes or terminals not accessible to the electrophysiological methods used.

J. *Mammalian-Like* ShIII *Genes Might Be a Recent Evolutionary Acquisition*

The coding sequence of one *Sh*III transcript (KV3.1a) has been sequenced in rodents (Yokoyama *et al.*, 1989; Ried *et al.*, 1993) and in humans (Ried *et al.*, 1993). In addition, sequence information is available on exon 2 of the human KV3.2 (D. Lau, E. Han, E. Vega-Saenz de Miera, and B. Rudy, unpublished) and KV3.3 (Lee and Roses, GenBank accession number Z11585) genes which can be compared with the corresponding sequences from KV3.2 and KV3.3 rodent cDNAs (T. McCormack *et al.*, 1990,1991; Luneau *et al.*, 1991b; Ghanshani *et al.*, 1992; Rudy *et al.*, 1992; Vega-Saenz de Miera *et al.*, 1992). The sequence of the constant region (prior to the divergent 3' ends) of KV3.4 in human (Rudy *et al.*, 1991b) and in rat (Schroter *et al.*, 1991) is also available. This partial comparative information can be used for a preliminary assessment of the degree of conservation of *Sh*III proteins throughout mammalian evolution (Table V). In all cases, the predicted proteins are highly conserved. For example, only a single amino acid difference is seen in the 511 residues of KV3.1a in rat and human. The degree of conservation of *Sh*III proteins is similar to that seen in the histones, the most conserved proteins known, and is indicative of very strong selection for the *differences* in structure and function of the protein products of the four *Sh*III genes. Conservation throughout the length of the protein is not required for assembly into functional channels since different *Sh*III proteins (with 70–80% amino acid identity) can form functional heteromultimeric channels. The conservation of the amino domain of KV3.4 could be associated with preserving the inactivation properties determined by this domain (Section IV,G). However, the consequences of the conservation of other portions of polypeptides that are not conserved among different members of the subfamily (i.e., the sequence C-terminal to the sixth membrane-spanning domain in KV3.1a, and the sequence of the linkers between the first and the second and the second and the third membrane-spanning domains in the four genes) are less clear, but may signify that these regions are also

TABLE V **Fraction of Identical Amino Acids in Analogous Positions in Pairs of Human and Rodent *Sh*III K^+ Channel Proteins**[a]

	KCNC2 (exon 2)	KCNC3 (exon 2)	KCNC4	Kcnc1	Kcnc2	Kcnc3	Kcnc4
KCNC1	.80	.82	.75	.99	.76	.77	.76
KCNC2 (exon 2)		.79	.84	.78	.99	.79	.82
KCNC3 (exon 2)			.78	.82	.76	.99	.78
KCNC4				.75	.74	.74	.97
Kcnc1					.76	.77	.76
Kcnc2						.72	.75
Kcnc3							.73

[a] Human genes are capitalized, rodent genes are lower cased.

important for specific roles of the protein products of each *Sh*III gene.

The functions that are being preserved are probably not merely the electrophysiological properties of the channels. For example, strong interspecific conservation is seen in KV3.1 and KV3.2 proteins, but both express currents that are very similar in voltage dependence, kinetics, and pharmacology in *Xenopus* oocytes. Additional comparative studies may shed some light on the nature of the functions that are being preserved. Alternatively, these proteins could be functionally identical, but their expression could be spatiotemporally distinct. Thus, both genes could be maintained only to allow for the expression of certain K^+ channels through mutually exclusive developmental pathways. However, the preliminary comparative analysis shows that the *differences* between the two proteins are conserved among species, implying that areas with gene-specific sequence encode functionally distinct domains. Information on the conservation of the divergent 3′ ends, now almost completely lacking (except for the carboxyl end of KV3.1a), may also be enlightening. The conservation seen to date suggests that, after duplication and subsequent variation, each one of the *Sh*III gene products seen in mammals has been incorporated into an important function that cannot be substituted by another member of the same subfamily.

As described in the introduction, mammalian products of a given *Sh* subfamily are more similar to one of the four *Shaker*-related genes in *Drosophila* than to a mammalian product of a different subfamily, indicating that precursor genes to each subfamily existed prior to the divergence of chordates and arthropods. *Sh*III proteins are more similar to the products of the *Drosophila Shaw* gene (Butler *et al.*, 1989) than to other *Drosophila* or mammalian *Sh* proteins (Yokoyama *et al.*, 1989; T. McCormack *et al.*, 1990,1991; Luneau *et al.*, 1991a,b; Rudy *et al.*, 1991a,b,1992; Schroter *et al.*, 1991; Ghanshani *et al.*, 1992; Vega-Saenz de Miera *et al.*, 1992). However, the percentage of amino acid identity seen between mammalian *Sh*III proteins and *Shaw* (49–56%) is less than that seen between mammalian and fly homologs in the other three subfamilies (70–80%) (Yokoyama *et al.*, 1989; T. McCormack *et al.*, 1990; Luneau *et al.*, 1991a,b; Rudy *et al.*, 1991b; Schroter *et al.*, 1991; Ghanshani *et al.*, 1992; Vega-Saenz de Miera *et al.*, 1992). Perhaps even more significant is the fact that, although *Sh*III proteins are more similar to *Shaw* in certain regions of the protein, they are more similar to members of other *Sh* subfamilies in the so-called S4 domain and in the sequences near this domain (Table VI). Consistent with this feature, the channels expressed by mammalian *Sh*III cDNAs in *Xenopus* oocytes differ considerably more from those expressed by *Shaw* than do members of the other subfamilies from their *Drosophila* homologs (compare mammalian *Sh*III currents described in Section IV,D with those expressed by *Shaw;* (Wei *et al.*, 1990).

The mechanism of evolution of mammalian-like *Sh*III subfamily genes may have been different from that of the genes in the other subfamilies. Assuming that mammalian-like *Sh*III genes are the homologs of the *Shaw* gene, several mechanisms can be postulated. If the hypothetical precursor of mammalian-like *Sh*III genes and *Shaw* resembled mammalian-like *Sh*III genes, *Shaw* might have arisen by accelerated divergence in the evolutionary line leading to arthropods. Alternatively, if the precursor resembled *Shaw*, mammalian-like *Sh*III genes may have evolved by parallel evolution or gene conversion events. The

TABLE VI **Amino Acid Conservation of Several Domains of *Sh* Proteins**[a]

Fraction of identical residues in KV3.2 and *Sh*K^+ channels of several subfamilies

Protein domains	KV3.2/*Shaw*	KV3.2/KV2.1	KV3.2/KV1.1
NH_2 domain[b]	0.67	0.47	0.34
S1	0.55	0.36	0.55
S2	0.55	0.50	0.41
S3	0.43	0.48	0.38
S4	0.45	0.50	0.64
S4–S5 linker	0.50	0.64	0.57
S5	0.55	0.45	0.45
H5	0.76	0.71	0.81
S6	0.91	0.68	0.82

Fraction of identical residues in rat and *Drosophila Sh*I and *Sh*II proteins

Protein domains	KV1.1/*Shaker*	KV2.1/*Shab*
NH_2 domain[b]	0.77	0.67
S1	0.77	0.73
S2	0.77	0.77
S3	0.81	0.71
S4	1.00	0.91
S4–S5 linker	0.93	0.86
S5	0.95	0.77
H5	0.86	0.71
S6	1.00	0.77

[a] The upper panel compares domains of KV3.2 proteins with the corresponding domains of *Shaw* (the fly homolog) and mammalian proteins of other subfamilies. The lower panel compares the same domains in fly and mammalian proteins of *Shaker* and *Shab* subfamilies.

[b] Refers to the area of the amino domain containing subunit recognition domains (see Section IV,G).

mosaic-like similarities of mammalian *Sh*III proteins and *Sh* genes of different subfamilies (Table VI) suggest gene conversion events. Whatever the mechanism, mammalian-like *Sh*III genes appear to be more specific to species in the evolutionary line leading to mammals. This factor may be important in considering possible functional roles of *Sh*III proteins, and justifies further studies on the evolution of these genes.

V. Summary and Perspectives

We have presented a discussion of the salient features of a subfamily of genes encoding putative subunits of voltage-gated K^+ channels; the *Shaw*-related or *Sh*III subfamily. Four *Sh*III genes have been identified, each generating multiple transcripts by alternative splicing. A total of 11 different *Sh*III cDNAs has been cloned. The various alternatively spliced transcripts from *Sh*III genes express voltage-gated K^+ channels in heterologous expression systems that activate at depolarized potentials more positive than −10mV. Transcripts of two genes (KV3.1 and KV3.2) express delayed-rectifier type currents whereas transcripts of the two other genes (KV3.3 and KV3.4) express A-type inactivating currents. *Sh*III currents can exhibit a wide range of inactivation properties as a result of the formation of heteromultimeric channels among different *Sh*III subunits. The specific but overlapping distribution of some *Sh*III mRNAs in rat brain, particularly the products of the KV3.1, KV3.3, and KV3.4 genes, lends credence to the idea of a system of heteromultimeric *Sh*III channels in the mammalian CNS. KV3.2 transcripts, which are expressed in many neurons that do not express transcripts of the other genes, may constitute a second independent channel system. Studies described in this chapter, particularly those localizing *Sh*III mRNAs to various tissues and neuronal populations in the CNS as well as those identifying candidate *Sh*III currents *in vivo*, represent important first steps in determining the exact relationship between cloned *Sh*III K^+ channel subunits and their *in vivo* correlates.

Perhaps the most important tools needed to pursue the identification of native *Sh*III channels in neurons and to gain insight into their functional role are subunit-specific antibodies. These antibodies are necessary to determine the localization of *Sh*III proteins to different regions of the neuronal membrane. The

mRNA expression studies discussed here suggest (see also Weiser *et al.*, 1994) the interesting possibility that native *Sh*III channels may vary in subunit composition from one neuronal population to another, with one subunit type dominating in one case and another dominating in another case. If we accept this possibility then, by extension, subunit composition in a given cell may change as a function of time, for example, throughout development or in response to activity or specific stimuli. In this manner, channel subunit composition could contribute to neuronal plasticity. Subunit-specific antibodies would be extremely useful to test these ideas. Antibodies could also be used to determine subunit composition of native channels immunoprecipitated from membrane extracts.

The identification of native *Sh*III channels to date has relied on finding similarities between the physiological properties of native channels and those of *Sh*III channels expressed in heterologous expression systems. Additional electrophysiological studies in heterologous expression systems, including determining the effects of second messenger cascades, will provide more clues that can be used to identify native *Sh*III channels and to understand the functional role of *Sh*III proteins *in vivo*. The goal of identifying native *Sh*III channels would be greatly facilitated, however, by the successful implementation of techniques that can specifically eliminate the currents expressed by a given *Sh*III channel. Such techniques would include novel channel-specific toxins as well as molecular techniques such as antisense hybrid arrest (Lotan, 1992) and gene knockout techniques in transgenic mice.

Studies on the regulation of expression of K^+ channel subunits in the nervous system could illuminate the longer term processes involved in its normal and pathological functions. Such studies would be without physiological context if we are uncertain of the nature of the currents produced by these subunits *in vivo*. This difficulty alone justifies the somewhat daunting task of identifying native *Sh* channels.

Moreover, the identification of channel proteins by cloning, in conjunction with approaches that allow the identification of the *in vivo* channels containing those proteins, can lead to the discovery of new, previously undescribed channels. This possibility is not surprising considering the current limitations of electrophysiological techniques, including the existence of electrophysiologically inaccessible neuronal processes and pre- and postsynaptic terminals. Studies with cloned channels *in vitro* may also lead to the discovery of new forms of K^+ channel function and modulation, as in the discovery by Ruppersberg *et al.* (1991b), described earlier in this chapter, that K^+ channels may be modulated by the oxidation of cysteine residues.

Note added in proof: (1) Attali *et al.* (1993a) have proposed that the I_{sK} protein may not be a channel but rather an activator of endogenous K^+ or Cl^- channels present in *Xenopus* oocytes. (2) Sheng *et al.* (1993) and Wang *et al.* (1993) have shown coprecipitation of two different proteins of the Shaker subfamily from solubilized membrane extracts in the presence of an antibody against only one of the proteins. These results are strong evidence that heteromultimeric channels exist *in vivo*. (3) Phorbol esters that activate protein kinase C have been shown to block inactivation of KV3.4 channels presumably as a result of phosphorylation of serines or threonines in the N-terminal insert mediating N-inactivation of these channels (Vyas *et al.*, 1993). (4) The existence of mRNA isoforms generated by alternatively-splicing has now been demonstrated as well for a mammalian Shaker homolog (Attali *et al.*, 1993b) and for a mammalian Shab homolog (D. Lau and B. Rudy, unpublished observations).

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: D. James Surmeier, *et al.* Group No.: 1635
Serial No.: 10/761,557 Examiner: Chong
Filed: 01/21/2004
Entitled: **Manipulation of Neuronal Ion Channels**

AMENDMENT TRANSMITTAL

Mail Stop Amendment
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CERTIFICATE OF MAILING UNDER 37 CFR § 1.8(a)(1)(i)(A)
I hereby certify that this correspondence (along with any referred to as being attached or enclosed) is, on the date shown below, being deposited with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Date: July 9, 2007 By: _____
Mary Ellen Waite

Sir or Madam:

Transmitted herewith is an amendment for this application. The fee has been calculated as shown below:

	Claims Remaining After Amendment		Highest Number Previously Paid For	Present Extra		Rate	Additional Fee
Total Claims	2	–	16	0	X	$50.00	$0.00
Independent Claims	1	–	4	0	X	$200.00	$0.00

SUBTOTAL:	$ 0.00
One-Month Extension of Time:	$ 120.00
SUBTOTAL	$ 120.00
Small Entity 50% Reduction	$ 60.00
TOTAL AMOUNT DUE:	**$ 60.00**

1. A check in the amount of $60.00 for an extension of time is attached.
2. Petition for extension of time. The undersigned attorney of record hereby petitions for an extension of time pursuant to 37 CFR § 1.136, as may be required to file this response.
3. The Commissioner is hereby authorized to charge any deficiency in the payment of the required fees, and/or credit any overpayment, to **Deposit Account No. 08-1290. An originally executed duplicate of this transmittal is enclosed for this purpose.**

Date: July 9, 2007

_____
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